Questing for Tick-borne Encephalitis virus in Belgium using Veterinary Sentinel Surveys and Risk factor Mapping

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Front cover illustrations:
- Iceberg: http://areteschola r.org/tag/iceberg/
- Tick warning sign: http://www.globalexterminating.com/ticked-off-lyme-disease-awareness-month/
- Epidemiological triad: https://onlinecourses.science.psu.edu/stat507/node/25

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- Questing female tick: https://www.wageningenur.nl/en/show/Tickhost-interactions.htm

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DEDICATION

This PhD is entirely dedicated to my parents

Dr. Patrik Roelandt and Mevr. Catheline Roelandt-Verstraete

The two bravest, smartest and kindest human beings I have ever known

and

without whom I would never have made it to this stage

of my veterinary and scientific career or in daily life …

Endless gratitude also to my sister Stéphanie and my "Bomma" !

Thanks to all my family members, colleagues and friends

who have remained faithful throughout time

and occasionally through difficult times …

Sophie - 17/06/2016

EPIGRAPH

Questing

1. The act or an instance of seeking or pursuing something - a search

2. A journey requiring great exertion, long travels and the overcoming of many obstacles

3. An expedition undertaken in medieval romance by a knight in order to perform a prescribed feat: e.g. the quest for the Holy Grail, the quest to destroy the One Ring (Lord of the Rings).

4. Archaic: an inquest or a jury appointed to take part in an inquest

5. from Middle English "queste", from Old French and ultimately from Latin "quaesta" (feminine of quaestus, the past participle of quaerere, to seek)

6. Tick climbing up a blade of grass or other structure and then waiting with its front legs outstretched to come into contact with a suitable host

The Quest
The Impossible Dream

Musical: Man of La Mancha – Don Quichote
Composer: Mitch Leigh - Songwriter: Joe Darion - Song Version: Andy Williams

To dream the impossible dream
To fight the unbeatable foe
To bear with unbearable sorrow
To run where the brave dare not go

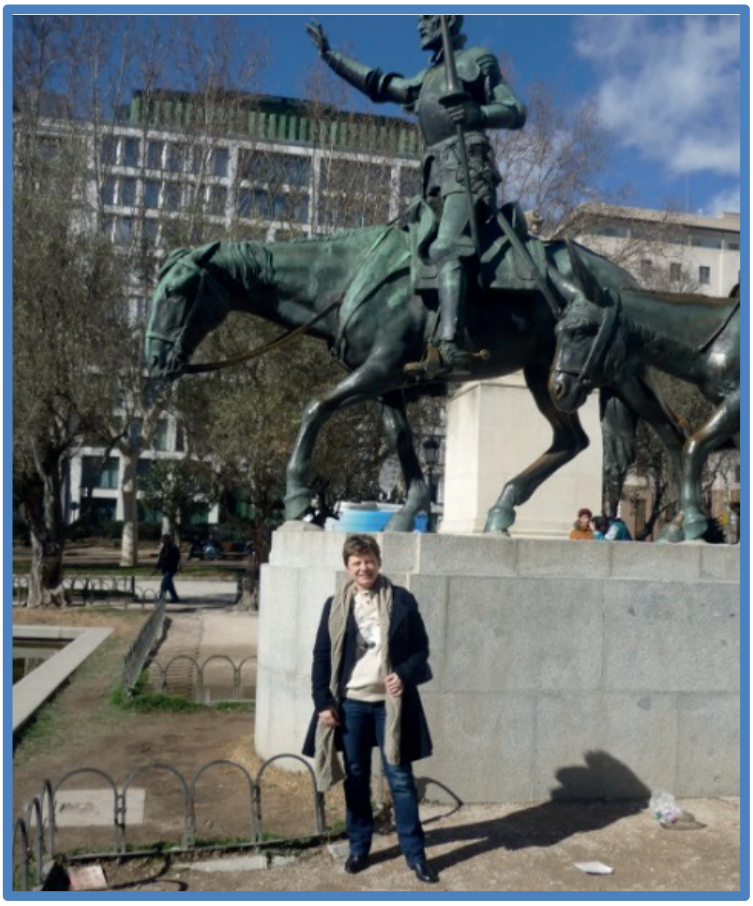
To right the unrightable wrong
To love pure and chaste from afar
To try when your arms are too weary
To reach the unreachable star

This is my quest, to follow that star,
No matter how hopeless, no matter how far
To fight for the right without question or cause
To be willing to march into hell for a heavenly cause

And I know if I'll only be true to this glorious quest
That my heart will lie peaceful and calm
when I'm laid to my rest

And the world will be better for this
That one (wo)man scorned and covered with scars
Still strove with his (her) last ounce of courage
To fight the unbeatable foe, to reach the unreachable star…

Dr. S. Roelandt with Don Quichote
La Mancha, Spain, 2015



Don Quixote

An ingenious, good-natured and slightly insane idealist who goes to battle on his exhausted old horse "Rocinante", with rusty armour, a paper helmet and a bean stalk as a jousting lance, to fight wind mills and other formidable enemies during his medieval quest for chivalrous romance

DANKWOORD

PhD staat voor 'Doctor of Philosophy'
(Wikipedia)

Filosofie mag hier in de brede zin geïnterpreteerd worden, volgens de originele Griekse betekenis **φιλοσοφία**: "liefde voor de wijsheid". Het doctoraats-diploma wordt door Europese universiteiten al sinds de middeleeuwen uitgereikt wanneer een kandidaat bewezen heeft problemen te kunnen aanpakken door vraagstelling, kritisch nadenken, een systematische aanpak en rationele argumenten (tot zover Wikipedia).

Per toeval is σοφία nu net mijn naam → dat creëert redelijk hoge verwachtingen voor de φιλο. En laat een PhD hedentendage nog steeds een soort **middeleeuwse queeste** zijn voor de doctorandus/-a, met als climax een verdediging van een proefschrift, waarvan het maken het gevecht met de draak zou kunnen evenaren. Je ziet direct dat voor deze hachelijke onderneming een flinke dosis filosofie vereist is, maar een goed harnas (tegen peer review), een bewezen strijdros (promotor) en geschikte wapens (software en geld) zouden zeker niet misstaan, ondanks de gesofisticeerde 21e eeuw waarin we leven...

Ik heb dan ook niet enkel veel over **TBEV** en labo's geleerd, maar ook over verschillende vormen van filosofie: de antropologishe (wat voor een beest is de mens?), de politieke (verdeling van macht, van kansen en mogelijkheden in een samenleving of organisatie) en de sociale (Human Behaviour = f(Person, Environment, Context). Aangezien een PhD onvermijdelijk uitmondt in een existentiële crisis (Waarom doe ik dit doctoraat? Waarom ben ik begonnen aan deze queeste van constante uitdagingen?) is het existentialisme ook van pas gekomen gedurende de laatste jaren. Hier en daar een snuifje dialectiek is ook nuttig gebleken om de redenaars-balans tussen **logos, ethos en pathos** verder te optimaliseren.

Om deze filosofische beschouwingen af te ronden, een citaat dat in deze tijden van zoönosen en one health toestanden zijn pluimen nog niet verloren is: "**Le sort des animaux est d'une plus grande importance pour moi que la crainte de paraître ridicule; il est indissolublement lié au destin des hommes**" (Émile Zola, 1840-1902). Zola volgde de literaire stroming van het naturalisme, die werd beïnvloed door het realisme en de ideeën van Auguste Comte, Karl Marx en Charles Darwin.

If you want to go fast, go alone. If you want to go far, go together.
(Afrikaans spreekwoord)

Ik ben natuurlijk niet op mijn eentje tot bovenstaande filosofische inzichten (en proefschrift!) gekomen. Vooreerst wil ik mijn sterk gewaardeerde **(co-)promotoren** Prof. Dr. Yves Van der Stede, Dr. Stefan Roels en Prof. Dr. Steven Van Gucht hartelijk bedanken voor hun onuitputtelijke toewijding en steun. Bedankt om mij de juiste weg te wijzen (doctoreren voor dummies die geen tijd hebben) en me gedurende al die jaren zowel op persoonlijk als op wetenschappelijk vlak verder te laten ontplooien. Na al die jaren van interessante informele discussies, labotesten, data verwerken, schrijven en herlezen mag het resultaat hopelijk gezien worden!

Hartelijk dank aan alle gedistingeerde leden van de **Begeleidings- en Examencommissies**: Prof. Dr. Hans Nauwynck, Prof. Dr. Jeroen Dewulf, Prof. Dr. Edwin Claerebout, Dr. Muriel Vervaeke, Dr. Guy Hendrickx, en Dr. Martin Beer. Uw gezamenlijk werk en opbouwende kritiek hebben significant bijgedragen tot de kwaliteit van dit proefschrift. U heeft mijn dankbaarheid in de vorm van een goede fles wijn en receptie zeker verdiend !

Dank ook aan het **CODA-CERVA**, om een wetenschappelijke omgeving te creëren met zeer goede collega's en waar doctoreren "on the job" nog niet als compleet geschift beschouwd wordt. Bedankt aan al mijn collega's die op gelijk welke manier bijgedragen hebben aan dit werk: alle wetenschappers en co-auteurs, laboranten en ook het ondersteunend personeel dat aan mij een hele kluif heeft. Een welgemeende dankuwel aan "Word-Wizard" Virginie en aan al diegenen die mijn die gezaag hebben moeten aanhoren in onze gezamenlijke bureau: Matthias, Valerie, Hanne, Mickaël en Genevieve. Ik heb ook altijd kunnen rekenen op Estelle, Sarah, Marc, Flavien en Michel, Isabelle en Katia, mijn collega **ERASURV'**ers van wie ik enorm veel geleerd heb !

Verder wens ik ook een heel grote dankuwel uit te brengen aan Dr. Vanessa Suin, Sophie Lamoral, en Dr. Bernard Brochier van het **WIV-ISP** voor het vele werk in het laboratorium en voor de goede samenwerking gedurende dit doctoraat: mag het nog lang zo verder gaan ! Dank voor de goede samenwerking aan het **FAVV** (Dr. Jef Hooyberghs), **DGZ** (Dr. Mia Vanrobaeys) en collega's), **ANB** (Dr. Muriel Vervaeke), **AviaGIS** (Dr. Els Ducheyne) en het **INBO** (Dr. Jim Casaer). Dank ook aan de kleine huisdieren **Laboratoria** (Labo Bruyland, Kortrijk; Laboratoires Jean Collard, Liège; Mediclab, Ghent), zonder wie de honden-studie onmogelijk was geweest.

Zonder uw medewerking en hulp was het allemaal heel wat minder geweest,
jullie hebben mee gezorgd voor deze taart én de kersen erop.

We mogen hier zeker ook de **familie en vrienden** niet vergeten, eigenlijk teveel mensen om op te noemen, maar bij wie we wel altijd terecht kunnen voor de nodige ontspanningsmomenten en voor informatie over wat een "normaal" leven behelst. Wees gerust, ik blijf tussen al dat studeren en diploma's halen ook nog tijd maken voor jullie! Een speciale vermelding nog voor Steef (mijn kleine zusje en soulmate Stéphanie) en Super-Bomma die altijd paraat staat.

Finaal moet ik hier ook veel snoepjes uitdelen aan mijn "**3 spin doctors**". Het zijn tenslotte mijn drie kattige huisgenoten Trinity, Macy en Felien, die het Don Quichotiaanse gezwoeg en geploeter van dag tot dag opgevolgd hebben en die mij al die jaren "geïnspireerd" hebben met hun filosofisch geluier op mijn PC en papieren.

At the end of the day, the most overwhelming key to a child's success is the positive involvement of its parents (Jane D. Hull, Amerikaans politicus).

En het blijft een waarheid als een koe, ook als je al halverwege de 30 aan het doctoreren bent... Daarom, last but not least, eeuwige dank aan mijn ouders aan wie ik dit werk ook opdraag.

Ik draag het op aan mijn **Moeder Catheline:** Zij die mij als baby vertroetelde, als kind mijn huiswerk opvroeg, als tiener op en af naar school voerde, tijdens mijn studententijd zorgde dat ik voldoende bananen en cola had tijdens de blok, die urenlang met mij telefoneerde toen ik in Engeland zat, en die mij op tijd en stond een hart onder de riem heeft gestoken gedurende dit doctoraat. Bovenal is Zij ook verantwoordelijk voor het aanwakkeren van mijn ambities tot dat "laaiend vuur" dat iedereen zo goed kent. Dat datzelfde vuur binnenkort ook bij Haar mag heroplaaien !

En ik draag het zeker ook op aan mijn **Vader Patrik**, voor altijd de enige echte "Dokter Roelandt" in mijn hart... Hij die mijn jeugd zo zorgeloos en gelukkig heeft gemaakt; Hij die me heeft geleerd te klussen, te plannen, te structureren en te reizen; Hij die mij de nodige genetica en brains heeft meegegeven om de wetenschap in te duiken; Diegene met wie ik mijn geneeskundige passie deelde, zij het toegepast op andere beesten... Hij die zo menselijk was en toch zo onmenselijk uit ons midden werd weggerukt.

Voor jou is dit doctoraat en voor jou begin ik nu aan de **volgende queeste** (die tevens de jouwe was): meer veiligheid voor hulpverleners. We gaan deze keer nog grotere "windmolens" bedwingen en "impossible dreams" proberen te verwezenlijken, maar gelukkig heb ik uit jouw levensvoorbeeld en uit dit doctoraat de juiste wapens meegekregen: **Logos, Ethos en Pathos...**

CURRICULUM VITAE

Sophie Roelandt was born on the 20th of November in Izegem (Belgium). After her high school studies with majors in science and mathematics at the Onze Lieve Vrouw Ter Engelen Instituut (a.k.a. 't Fort, Kortrijk, Belgium), she commenced the study of Veterinary Medicine at Ghent University in 1997. She obtained the degree of Veterinary Surgeon (MSc) in 2003 with high distinction.

Sophie then spent 6 years in the United Kingdom, first in small animal practice and later conducting a small animal clinical internship at Liverpool University. This was followed by a second Masters in Wild Animal Health at the Royal Veterinary College and the Zoological Society of London (London, United Kingkom). This MSc WAH degree was obtained with merit in 2008 and was followed by a clinical internship at the Dubai Falcon Hospital and at the Sharjah Zoo and Arabian Wildlife Center (early 2009, United Arab Emirates).

In 2009 Sophie returned to Belgium to work as a scientific researcher at the Veterinary and Agrochemical Research Centre (CODA-CERVA), first in the Wildsurv Project and later at the epidemiological unit ERASURV. She is currently involved in laboratory test evaluation, risk assessments and surveillance of notifiable and zoonotic veterinary infectious diseases. This epidemiological research is conducted in close cooperation with the Belgian Federal Agency for the Safety of the Food Chain (FASFC-FAVV-AFSCA), and together with many other (inter)national and regional research institutes and universities.

Sophie obtained the Certificate of Veterinary Epidemiology at Ghent University in 2010, with high distinction. She is currently a member of the Belgian College of Veterinary Surgeons (NGROD No. 3904), a Member of Belgian Wildlife Disease Society (BWDS), vice-president of the Flemish Society for Veterinary Epidemiology & Economics (FSVEE), and a resident of the European College for Veterinary Public Health (ECVPH).

As part of her government research work (2009-2016), Sophie has been involved in the epidemiological design and analysis of three studies on Tick-borne Encephalitis Virus (TBEV) surveillance in sentinel animal species. The methods and results of these studies are the subject of this PhD thesis. Sophie Roelandt is author and co-author of multiple scientific publications on TBEV and other veterinary epidemiology topics. On several occasions she has presented and illustrated her research results at (inter)national scientific conferences.

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FIGURES

TABLES

ABBREVIATIONS

CFT	Complement fixation test
CODA-CERVA	Belgian Federal centre for veterinary and agrochemical research
CSFV	Classical swine fever virus
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	Computer tomography
DSe	Diagnostic sensitivity of a laboratory test
DSp	Diagnostic speciticity of a laboratory test
EIA	Enzyme immuno-assay
ECDC	European Center for Disease Prevention and Control
e.g.	*exempla gratia* = for example
ENIVD	European Network for Diagnostics of Imported Viral Diseases
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
FE-TBEV	Far Eastern TBEV subtype
FLI	Friedrich Loeffler Institute, Germany
FSME	Früh sommer meningo-encefalitis = TBE
GGEV	Greek goat encephalitis virus
i.e.	*id est* = meaning
IFA	Immunofluorescent assay
IgM or IgG	Immunoglobulin/antibody of isotype M (acute) or G (subacute)
ISW-TBE	International Scientific Working Group for TBE
ITM	Belgian Institute of Tropical Medicine
LFD	Lateral flow device
LIV	Louping Ill virus
MIT	Mouse inoculation test
MS	Member State (of the European Union)
MWU-test	Mann-Whitney-U test: nonparametric comparison test
NIS	National institute of statistics codes for Belgian municipalities
NPV	Predictive value of a negative test result
PPV	Predictive value of a positive test result
RKI	Robert Koch Institute, Germany
RT-PCR	Reverse-transcriptase polymerase chain reaction
SNT	Seroneutralisation test (syn. vironeutralisation test)
spp.	Multiple species
SGEV	Spanish goat encephalitis virus
SSEV	Spanish sheep encephalitis virus
TBEV	Tick-borne encephalitis virus
TSEV	Turkish sheep encephalitis virus
UTM squares	Universal transverse Mercator coordinate system
VBD	Vector-borne disease
W=Eu-TBEV	Western = European TBEV subtype
WIV-ISP	Belgian Federal Scientific Institute of Public Health

CHAPTER I GENERAL INTRODUCTION: TICK-BORNE ENCEPHALITIS EPIDEMIOLOGY, LABORATORY DIAGNOSIS AND EPIDEMIOLOGICAL SURVEILLANCE

Adapted from:

S. Roelandt, P. Heyman, P. Tavernier, S. Roels

TICK-BORNE ENCEPHALITIS IN EUROPE:

REVIEW OF AN EMERGING ZOONOSIS.

Vlaams Diergeneeskundig Tijdschrift (2010), 79: 23-31.

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I.1 BACKGROUND

Tick-borne encephalitis virus (TBEV) is the most important arthropod-borne virus in Europe (Herpe et al., 2007; Ramelow et al., 1993). In Europe, the Western subtype of this highly pathogenic neurotropic flavivirus is carried by *Ixodes ricinus* (Kreil et al., 1997; Labuda and Randolph, 1999; Stjernberg et al., 2008). Tick-borne encephalitis (TBE) has become a considerable public health risk in several European countries (Haglund, 2002; Süss et al., 1997), with currently on average 3,000 hospitalized cases per year (ECDC, 2012; Mansfield et al., 2009; Süss, 2011). In many patients the disease results in long-term sequelae and disability (Donoso Mantke et al., 2008a).

Recent increases and fluctuations in human incidence in Central and Eastern European countries (e.g. Switzerland, Germany, Poland, Baltic States) (Süss, 2008a, b) and the emergence of the disease in Finland (Jääskeläinen et al., 2006), Norway (Csángó et al., 2004); (Skarpaas et al., 2006; Skarpaas et al., 2004), Denmark (Skarpaas et al., 2006; Skarphedinsson et al., 2005) and France (Herpe et al., 2007) have sparked international concern and research. TBE is also emerging in Europe's canine population, and the numbers of clinical cases in dogs are expected to increase (Beugnet et al., 2009; Leschnik et al., 2002).

This review aims to highlight important features of TBE epidemiology, the clinical course in humans and the surveillance possibilities for this tick-borne flavivirus. Afterwards, it will enter the discussion of whether Belgium could be at risk for TBE and whether national veterinary surveillance, in addition to medical surveillance, could be of benefit.

I.2 EPIDEMIOLOGY

I.2.1 EPIDEMIOLOGICAL CONCEPTS

Since the early days of recognition of TBE disease in the early 20th century, it has been known that the ecology and epidemiology of TBEV is a complicated story (Figure I-1) (Pfeffer and Dobler, 2011). As with most infectious diseases, the complexity of the real life situation may be reduced and simplified with the help of the classic epidemiological concept of the **epidemiological triad:** agent – host – environment + vector (Figure I-1). A host and an external agent are brought together in an environment, causing the disease in the host. A vector may transmit from one host to another (Lengerich, 2016).

Hence, we can review the recent advances in a large part of TBE research from these different eco-epidemiological viewpoints that are connected by the vector. Of all separate parts of the epidemiological triad and of the multifactorial drivers that contribute to the TBEV transmission cycle, our understanding continues to increase (See I.2.2- I.2.7).

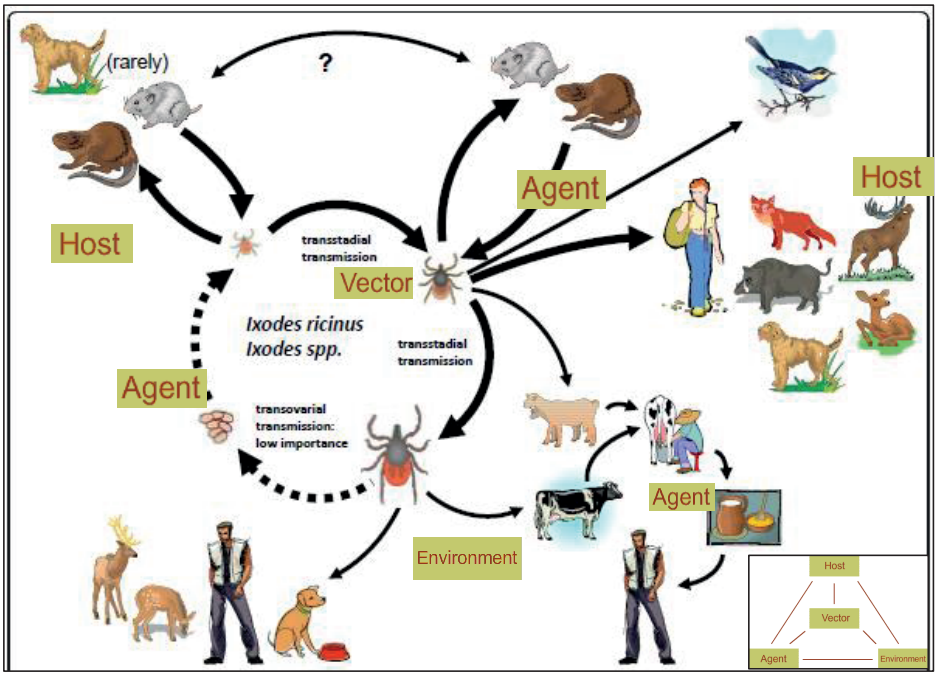

Figure I-1 : Transmission cycle of TBEV with parts of the epidemiological triad.
Agent - Host - Environment – Vector; Adapted from Pfeffer and Dobler, 2011 and Lengerich, 2016

The **zoonotic iceberg** (Figure I-2) is another analogy which helps to explain such complicated observed variability in human TBE incidence. It involves investigating the lower parts of bulk of the iceberg that are invisible and submerged, i.e. domestic animal exposure and the much more extensive endemic wildlife transmission cycles between vectors and wildlife hosts, in order to explain the variable human infections and cases at the visible tip of the iceberg..

In other words, the bulk represents the transmission potential of a risk area, while the tip represents the human exposure and contact rates with TBEV through ticks and the environment (Randolph and Sumilo, 2007). It is very important to select the right part of the iceberg for the right kind of surveillance (See under I.5 and VII.1-2).

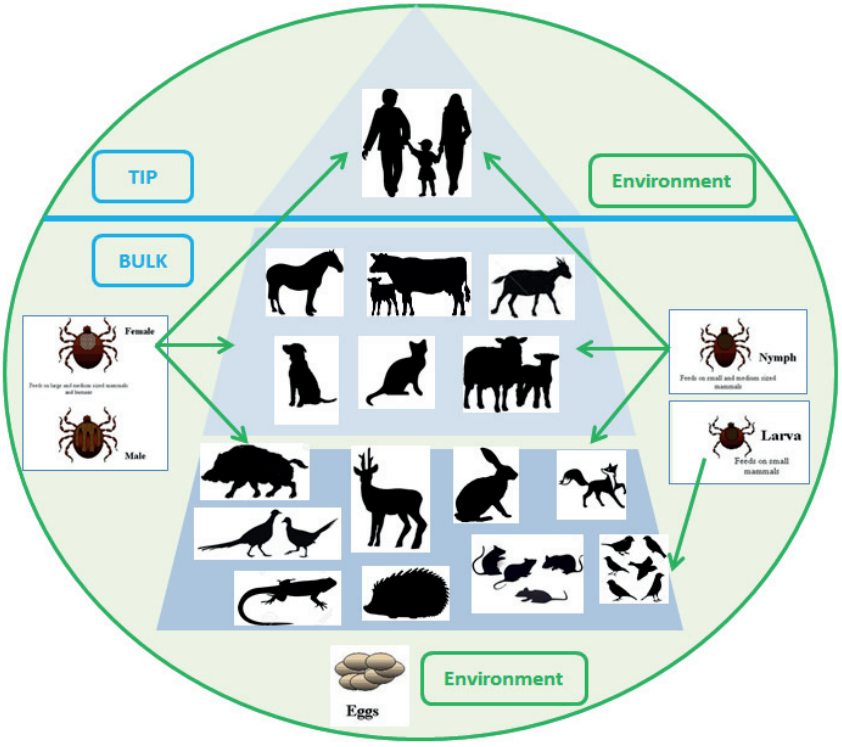

Figure I-2: The Zoonotic Iceberg Analogy
Created based on (Randolph and Sumilo, 2007) and (Liverpool, 2015)

I.2.2 PATHOGENIC AGENT: TBEV

TBEV is a small spherical enveloped RNA virus that belongs to the genus Flavivirus (Flaviviridae), which contains many neurotropic pathogenic arthropod-borne viruses. Its genome consists of a 10.5kb single positive strand of RNA, encoding three structural and seven non-structural proteins (Gould et al., 2004; Heinz, 2003; Heinz and Allison, 2000). Three lineages of TBEV have been described, namely the Western subtype (W-TBEV) transmitted by *I. ricinus* ticks and the Siberian (S-TBEV) and Far Eastern (FE-TBEV) subtypes transmitted by *I. persulcatus* (Donoso Mantke et al., 2008a; Gubler et al., 2007). Besides the co-circulation of all subtypes in Estonia and Latvia (Golovljova et al., 2004) and one focus of the Siberian subtype discovered in Finland (Jääskeläinen et al., 2006), only the Western subtype is present in Europe (Heinz, 2008). Hereafter, only the Western subtype will be discussed, unless specified otherwise.

I.2.3 TICK VECTORS

The castor-bean syn. sheep tick *I. ricinus* (Family Ixodidae) is the most important and most widespread European tick species (Figure I-3) (VBORNET, 2015) and it was long believed to be the only tick in nature capable of sustaining infection with the Western TBEV (W-TBEV) subtype (Heinz, 2008; Labuda and Randolph, 1999).

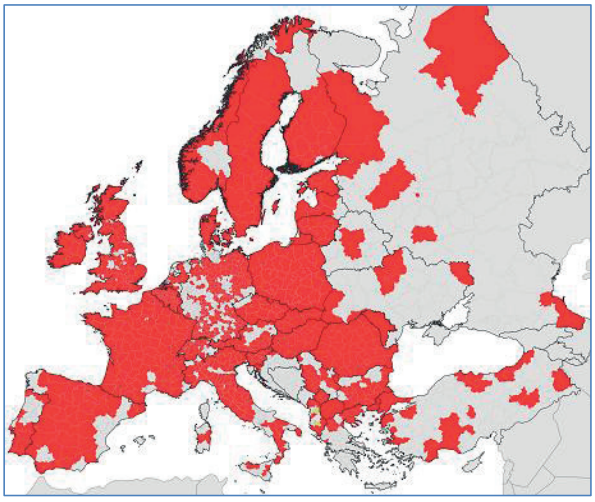

Figure I-3. Distribution of *I. ricinus* ticks in Europe.
Red zone: presence of I. ricinus in the area; Grey zone: no data or no presence (VBORNET, 2015)

I. ricinus is a three-host tick with three life stages (larva, nymph, adult) and a two/three-year-long life cycle during which TBEV infection is passed transstadially, horizontally, and transovarially/vertically (Figure I-4a). TBE infected ticks can be co-infected with many pathogens, but mainly with *Borrelia spp., Babesia spp., Anaplasma spp.* or *Coxiella burnetii* (Heinz, 2008; Süss, 2003). After the blood meal, TBE virus replication takes place in the tick's midgut wall cells. Infection of the salivary glands then occurs prior to transmission by tick saliva into the next host during the next blood meal, within 1-3 hours after attachment (Figure I-4a) (Gresikova and Noseck, 1966; Mansfield et al., 2009; Nuttall et al., 1994; Ruzek et al., 2013; Weitlauf et al., 2007).

Horizontal tick-to-tick transmission may thus occur through mating, through viremic hosts, or through simultaneous co-feeding of larvae with nymphs on the same (immune) host (Figure I-4b) (Donoso Mantke et al., 2008a; Heinz, 2008; Labuda et al., 1993a; Labuda and Randolph, 1999; Mansfield et al., 2009; Vene et al., 1998). Transmission to the host happens (1) via Langerhans cell infiltration/migration in local host skin (Figure I-4c)(Labuda et al., 1996), or (2) via saliva mixing in the same feeding pool (Figure I-4d) (Alekseev and Dubinina, 2002; Nuttall et al., 1994; Ruzek et al., 2007a) or (3) through the host's viraemia (Süss, 2011). The *I. ricinus* transmission of W-TBEV as well as their infection level of these ticks is still being studied and becoming better understood (Biernat et al., 2014b; Hubalek and Rudolf, 2012; Stefanoff et al., 2013).

It is known that the ornate dog tick *Dermacentor reticulatus* (Amblyommidae) is also involved in the circulation of TBEV; however, its relative importance for TBE epidemiology remains unknown (Biernat et al., 2014b). When experimentally infected with W-TBEV, *D. reticulatus* shows virus proliferation and TBEV transmission to a new host within 1 hour of attachment (Alekseev et al., 1996; Rehacek et al., 1987), but it seems to exhibit lower transmission rates (Gresikova and Kaluzova, 1997).

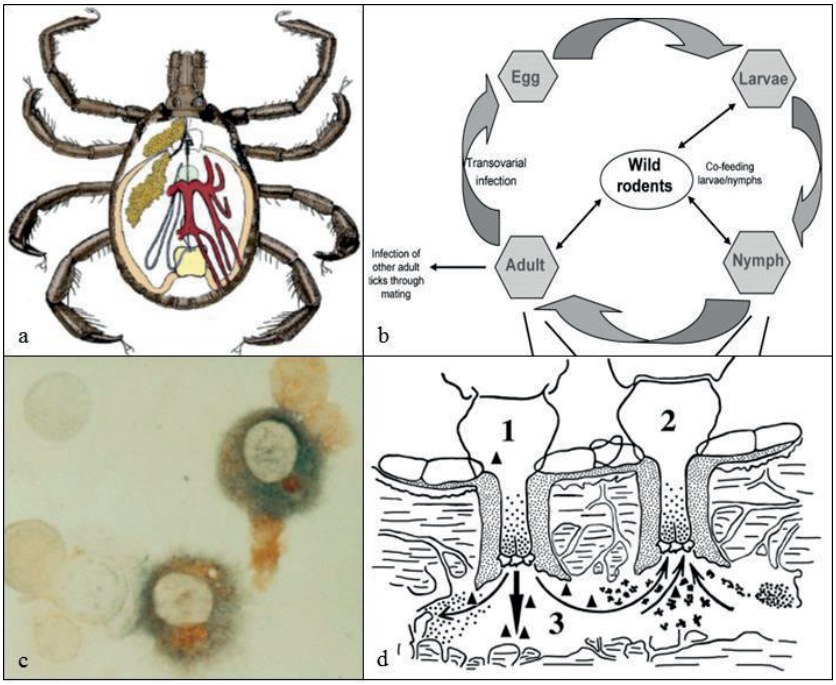

Figure I-4: Tick-to-tick ransmission of tick-borne encephalitis virus
(a) Important tick anatomy for TBEV transmission: mouth parts (chelicerae and hypopharynx), salivary glands, midgut (Edwards et al., 2009); (b) Transmission throughout the tick life cycle (Mansfield et al., 2009); (c) Cellular co-feeding transmission - Immunocytochemical localization of TBE viral antigen (red) in MHC class II-positive Langerhans cells in mouse skin (blue) (Labuda et al., 1996); (d) Trans-salival co-feeding transmission - 1: donor, 2: recipient,3: feeding site (Alekseev and Dubinina, 2002).

I.2.4 VERTEBRATE HOSTS

I. ricinus has more than 100-300 known natural hosts, including mammals, birds and reptiles. Most of these hosts become subclinically infected with TBEV and become immune for life (Leschnik et al., 2002; Süss, 2003; WHO, 2011). *D. reticulatus* also occurs widely in the temperate climate zone across Eurasia, parasitizing a wide range of host species (Biernat et al., 2014b) and its distribution is currently expanding in north-western Europe (Karbowiak and Kiewra, 2010).

Small rodents such as field and wood mice (*Apodemus* spp., *Myodes* spp.) show asymptomatic viremia and constitute competent reservoirs for immature ticks (Cerny, 1975; Heinz, 2008; Süss, 2003), with rapid population turnover and a constant supply of susceptible individuals (Rizzoli et al., 2004a). Additionally, TBEV is able to replicate in immunocompetent (Langerhans) cells in the skin of these mice, including immune or aviremic animals. The infected cells migrate to lymph nodes and back to feeding sites of uninfected co-feeding ticks. This allows non-viremic transmission between simultaneously co-feeding nymphs and larvae, even in immune rodents (Labuda et al., 1993d; Labuda and Randolph, 1999).

Large mammals such as roe deer (*Capreolus capreolus*), wild boar (*Sus scrofa*) and domestic ruminants mainly serve as tick amplifying hosts. They are considered incompetent hosts (i.e. unable to transmit), as they only develop low virus titers, though non-viraemic co-feeding transmission may be possible (Labuda et al., 1993d; Rizzoli et al., 2004a; Süss, 2003). TBEV is excreted in the milk of viremic cows, goats and sheep and can be transmitted to humans this way (Gould et al., 2006; Heinz, 2008). Birds also host immature *I. ricinus* and experience a short TBEV viremia. They seem to contribute to the spread of infected ticks (Gould et al., 2006; Gould et al., 2004; Heinz, 2008).

Dogs and horses appear to be relatively less frequently infected by TBEV, though they may carry infected Ixodid ticks from endemic to non-endemic areas and into close vicinity of humans (Balmer et al., 2007a; Chomel, 2013; Gresikova et al., 1972; Higgins and Snyder, 2006; Leschnik et al., 2002; Rees, 2010). Although in most of these animals there is seroconversion without clinical signs (Grešíková et al., 1972; Janitza-Futterer, 2003; Klimeš et al., 2001; Leschnik et al., 2002; Rushton et al., 2013), TBEV can nevertheless cause general and multifocal neurological clinical signs (Bjöersdorff, 2002; Dietz and Huskamp, 2005; Kirtz, 1999; Müller et al., 2006). Humans are accidental dead-end hosts for ticks and for TBEV as, despite noticeable viremia, humans do not transmit the disease (Heinz, 2008; Süss, 2003).

I.2.5 ENVIRONMENT

Around 90% of TBEV endemic foci fall within specific climatic boundaries, such as the 7-8°C annual isotherm and the 800 mm per annum precipitation minimum, resulting in the high soil humidity and 92% relative humidity required by *I. ricinus* for survival (Gritsun et al., 2003b; Heinz, 2008; Labuda and Randolph, 1999). Infected *I. ricinus* are often found questing for a host in mixed deciduous woodland with dense, humid ground layers and in animal feeding and resting places (Heinz, 2008; Randolph, 2001).

Co-feeding of larvae and nymphs can only occur in areas where rapid autumnal cooling inhibits questing of unfed larvae, which will go into diapause until the next spring. At this time, rapidly rising temperatures will cause simultaneous reactivation of larvae and nymphs (Gritsun et al., 2003b; Randolph, 2001). Nymphal and larval seasonal feeding patterns will then overlap and this leads to co-feeding transmission (Labuda and Randolph, 1999).

Without co-feeding, TBEV does not seem to persist and an endemic focus should not develop (Gould et al., 2004; Randolph, 2002). No vertical transmission route can be sufficient without additional horizontal amplification. Virus passing from one female to 50% of her offspring will die out within a few generations, unless infected offspring have double the normal survival rate (Randolph, 2011).

Labuda's laboratory work on transmission pathways together with the quantitative features of tick survival are considerred sufficient to explain persistent TBE virus cycles (Labuda et al., 1996; Labuda et al., 1993a; Labuda et al., 1993b; Labuda et al., 1993c; Labuda et al., 1997b; Labuda et al., 1993d; Labuda and Randolph, 1999; Randolph et al., 1999).

In natural conditions, it seems that a reproduction ration (R0) above 1 (which is an indicator for ongoing transmission with possible outbreaks, only occurs when there is transmission of non-systemic infections from infected nymphs to larvae by co-feeding (Randolph et al., 1999; Hartemink et al., 2008).

I.2.6 Distribution and Incidence

The distribution of TBEV (all 3 subtypes) spans almost the entire Southern part of Eurasia (Figure I-5)(Barrett et al., 2008; Kollaritsch et al., 2011b; Süss et al., 2010), presently from Alsace-Lorraine/Southern Norway (Donoso Mantke et al., 2008a) to Vladivostok/Northeastern China/Japan, and it consists of up to 30,000 endemic foci (Gritsun et al., 2003b; Korenberg and Kovalevskii, 1999). Worldwide, 10,000-12,000 cases of human TBE are reported annually (Süss, 2003), of which more than 3,000 cases in Europe (Charrel et al., 2004; ECDC, 2012; Gritsun et al., 2003a; Gunther and Haglund, 2005; Haglund, 2002). European TBEV is endemic in 27 countries (Süss, 2008a), of which the Central European countries, the Baltic States and Russia are most severely affected (Korenberg and Kovalevskii, 1999; Süss et al., 1992).

In many of these countries TBE accounts for 50% of all cases of central nervous system (CNS) infection (Kaiser, 2008a; Kunz, 2008). Western European incidence is currently <4 cases/100,000 inhabitants (Gubler et al., 2007; Stjernberg et al., 2008). However, in Great Britain, Ireland, Iceland, Belgium, the Netherlands, Spain and Portugal no autochthonous TBE cases have been reported to date (Donoso Mantke et al., 2008a; ECDC, 2012; Kunz, 2008).

During the last three decades, the TBE incidence has risen dramatically (2- to 17-fold) in most of the affected countries (e.g. Lithuania 1033%, Germany 574%, Europe as a whole 400%) (Randolph and Rogers, 2000; Stjernberg et al., 2008; Süss, 2008a, b). Recently, new endemic foci have appeared in France, Greece, Denmark, Norway (Donoso Mantke et al., 2008a), Italy (Pugliese et al., 2007), Sweden (Stjernberg et al., 2008), Finland (Jääskeläinen et al., 2006) and Germany (RKI, 2009), and new risk areas are being discovered every year. TBE has rapidly become a growing public health problem (Süss, 2008a, b) and is currently the most important vector-borne viral infection in Europe (Donoso Mantke et al., 2008a; Labuda et al., 2006).

It is known that TBE risk areas in countries can be very large and static, but they can also be small and dynamic in time and space, with sudden dis- and re-appearances of human cases, even outside previously recognized endemic areas (Kupca et al., 2010; Randolph and Sumilo, 2007).

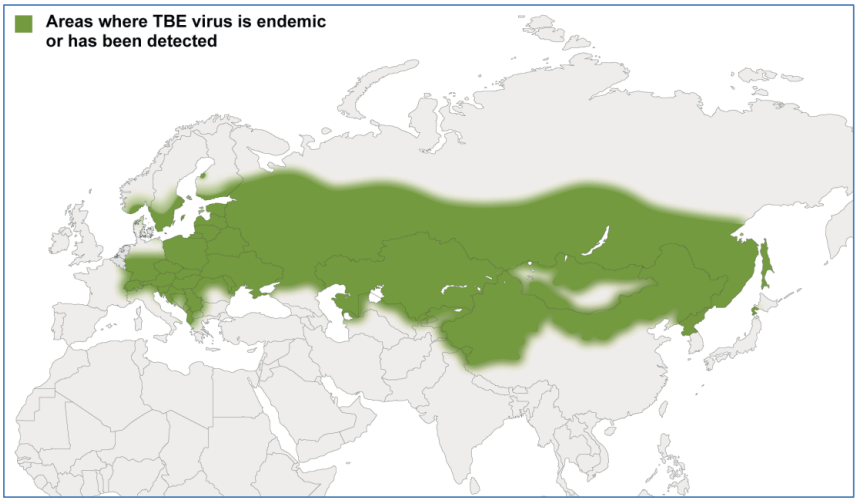

Figure I-5. Known distribution of the TBE virus
(Kollaritsch et all, 2011; Barrett et al., 2008; Süss, 2010).

I.2.7 DRIVERS AND RISK FACTORS

During the last 30 years, European TBE incidence trends were characterized by wild fluctuations, relative stability and gradual increases in different areas, while spatio-temporal endemic area distribution was patchy/variable (Nuttall, 1999; Randolph and Sumilo, 2007; Süss, 2011). Several authors discussed the many various sociological, technological and ecological (a)biotic[1] etiological risk factors that may cause a rise in incidence, prevalence and distribution of vector-borne diseases (VBDs), and specifically of TBE. (Figure I-6; Table I-1).

Whereas recent TBE emergence in Scandinavia has been linked predominantly to climate change (milder winters and earlier springs) (Lindgren and Gustafson, 2001), expansion of roe deer and tick populations (Randolph, 2001, 2008) and increased awareness (Haglund, 2002), increases in incidence in certain areas of Germany during the 1990s seem to be related to increased surveillance and improved diagnostics (Randolph, 2001). As opposed to this, in Eastern European countries the political change caused by the end of communism also resulted in many agricultural and social changes (Randolph, 2001).

[1] Living organisms and chemical/physical parts of the environment that affect living organisms and the functioning of ecosystems

As a consequence, the increased consumption of unpasteurized milk and the increased use of forests and previously abandoned countryside areas for food collecting or leisure activities have led to increased exposure to *I. ricinus* and TBEV (Beltrame et al., 2006; Randolph and Rogers, 2000). Finally, data indicate that some dramatic increase of human TBE cases in several European countries during hot summers can be explained mainly by an increase in human outdoor activity in response to the unusual weather (Randolph, 2004; Randolph et al., 2008).

Since risk factors such as changes in climate/weather, society, politics, public health actions, land use, wildlife abundance and human behavior can strongly influence TBE-incidence (Lindgren, 1998; Mansfield et al., 2009; Randolph, 2004; Randolph and Sumilo, 2007; Süss, 2008b), monitoring and evaluating these drivers may ultimately enable prediction of the multifactorial and heterogeneous changes in incidence and potential emergences of TBE as observed in the field (Jaenson et al., 2012; Randolph and Sumilo, 2007).

Changes in hunting practices have led to increasing European game populations, providing greater feeding opportunity for questing ticks (Sumilo et al., 2008b; Rizzoli et al., 2009; Rizzoli, 2009). Some authors have assumed that, since game are dead end hosts, this would lead to dilution effects and thus a decrease in TBE incidence (Kriz et al., 2014; Perkins et al., 2006; Rosa and Pugliese, 2007; Pugliese and Rosa, 2008; Cagnacci et al., 2012; Bolzoni et al., 2012).

However, this view did not take into account co-feeding non-viraemic transmission (Labuda et al., 1993a/b/c; Randolph et al., 1996; Randolph and Sumilo, 2007) or vertical/transovarial transmission (Kriz et al., 2014). Indeed, the increasing host abundance and extending distribution has led to enhanced tick reproduction with associated spread of TBEV to higher latitudes/altitudes and the emergence of new endemic foci outside woods in agricultural and suburban areas (Medlock et al., 2013; Jaenson et al., 2012; Gomez Martinez, 2014; Cisak et al., 2012/2014; Kriz et al., 2014).

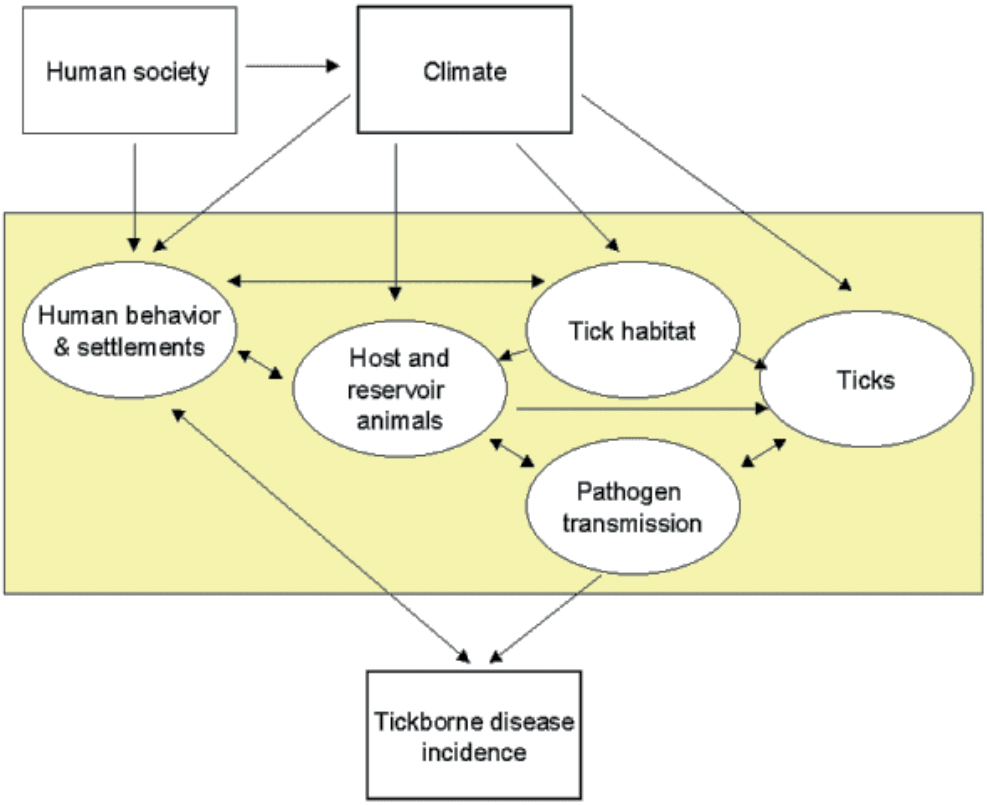

Figure I-6: Risk factors of tick-borne diseases.
Schematic relationship between several (a)biotic drivers; Lindgren, 1998.

Table I-1: Risk factors influencing incidence, prevalence and distribution of tick-borne encephalitis, ticks and Lyme disease	
Risk Factor	**References (non-exclusive)**
Increased human population and density - Increased urbanization and abandonment of countryside - Migration towards suburban areas	(Beltrame et al., 2006; Beugnet and Marie, 2009; Heinz, 2008; Linard et al., 2007; Linard and Vanwambeke, 2009; Merino et al., 2000; Racz et al., 2006; Randolph, 2001; Randolph and Sumilo, 2007; Süss, 2003; Süss, 2008a)
Exotic disease introduction through increased travel of humans/pets and increased transport of goods and domestic/wild animals	(Beugnet and Marie, 2009; BSAVA, 2009; Donoso Mantke et al., 2008a; Haglund, 2002; Heyman, 2009; Kunz, 2008; Leschnik et al., 2002; Luyasu, 2009; Rendi-Wagner, 2004; Süss, 2003; Süss, 2008a)
Change in leisure and outdoor activities	(Donoso Mantke et al., 2008a; Heinz, 2008; Kunz, 2008; Lindgren and Gustafson, 2001; Randolph et al., 2008; Randolph and Rogers, 2000)
Social/political/economic factors and change - Displacement due to conflict/war	(Beltrame et al., 2006; Beugnet and Marie, 2009; De Keukeleire et al., 2015; Donoso Mantke et al., 2008a; Heinz, 2008; Linard et al., 2007; Linard and Vanwambeke, 2009; Merino et al., 2000; Randolph, 2001; Randolph et al., 2008; Randolph and Rogers, 2000; Süss, 2003; Süss, 2008a; Vanwambeke et al., 2010; Zeimes et al., 2014)
Change in agricultural practices - Increased consumption of raw milk	(Heinz, 2008; Randolph, 2001; Randolph et al., 2000; Süss, 2003; Süss, 2008a)
Occupational exposure: farmer, military, forester, hunter, laboratory	(Donoso Mantke et al., 2008a; Randolph, 2001; Süss, 2003; Süss, 2008a)
Landscape structure, configuration, fragmentation, geology and land use	(Achazi et al., 2011; Beltrame et al., 2006; Beugnet and Marie, 2009; Brownstein et al., 2005; Bunnell et al., 2003; Daniel et al., 1998; Das et al., 2002; De Keukeleire et al., 2015; Dister et al., 1997; EEA, 2012; Falco et al., 1999; Glass et al., 1995; Guerra et al., 2002; James et al., 2013; Kiffner et al., 2010; Li et al., 2012a; Li et al., 2012b; Linard et al., 2007; Linard and Vanwambeke, 2009; Randolph, 2001; Randolph et al., 2008; Vanwambeke et al., 2010; Wilson et al., 1985; Zeimes et al., 2014)
Increased reforestation/conservation of tick habitat and changed hunting or wildlife management practices, leading to increasing host and tick populations	(Allan et al., 2003; Beugnet and Marie, 2009; Gómez-Martínez, 2014; Haemig et al., 2011; Haglund, 2002; James et al., 2013; Kiffner et al., 2010; Knap and Avsic-Zupanc, 2013; Kriz et al., 2014; Li et al., 2012a; Li et al., 2012b; Linard and Vanwambeke, 2009; Lindgren and Gustafson, 2001; Randolph, 2001; Randolph et al., 2008; Rizzoli et al., 2009; Stjernberg et al., 2008; Süss, 2008a; Wilson et al., 1985)
Climatological (climate change) or meteorological (local microclimate)	(Achazi et al., 2011; Beugnet and Marie, 2009; Brownstein et al., 2003; Daniel et al., 2011; De Keukeleire et al., 2015; Donoso Mantke et al., 2008a; EEA, 2012; Haglund, 2002; Heinz, 2008; Heyman, 2009; James et al., 2013; Labuda et al., 1997a; Labuda and Randolph, 1999; Li et al., 2012a; Li et al., 2012b; Lindgren and Gustafson, 2001; Lindh et al., 2008; Palo, 2014; Randolph, 2001; Randolph et al., 2000; Randolph and Sumilo, 2007; Rizzoli et al., 2007; Stjernberg et al., 2008)
Environmental factor: biotope, vegetation	(Beugnet and Marie, 2009; Estrada-Pena, 2002a; Haglund, 2002; Heinz, 2008; James et al., 2013; Linard et al., 2007; Randolph, 2001; Rizzoli et al., 2009; Stjernberg et al., 2008; Tack et al., 2012; Zeimes et al., 2014)
Higher awareness in the medical community and among the population in general	(Beltrame et al., 2006; Donoso Mantke et al., 2008a; Haglund, 2002; Lindgren and Gustafson, 2001; Randolph, 2001; Stjernberg et al., 2008)
Improved diagnostic procedures and increased surveillance	(Beltrame et al., 2006; Donoso Mantke et al., 2008a; Haglund, 2002; Randolph, 2001; Stjernberg et al., 2008)

I.3 CLINICAL CASES

I.3.1 EXPOSURE

Humans and animals usually become infected with TBEV through bites from *I. ricinus* larvae, nymphs or adults. In affected countries, people contract TBE during spring and summer through working outdoors (e.g. farmers, forest workers, military personnel) (Donoso Mantke et al., 2008a; Haglund, 2002) or through leisure activities such as sports, hunting, fishing, rambling/hiking, berry/mushroom/wood collecting, or through the consumption of raw (unpasteurized) goat, cow and sheep milk (Heinz, 2008; Süss, 2003; Süss, 2008a). The risk of human infection after a single tick bite in an endemic area varies from 1/1000 to 1/200 (Süss, 2003; Süss, 2008a).

I.3.2 CLINICAL COURSE

TBEV is neurotropic and clinical signs vary from mild fever to meningitis, encephalitis or myelitis (Haglund et al., 2003). Between one-third and two-thirds of human patients experience a typical biphasic course, as in Figure I-7 (RicHard-59, 2011), starting with non-specific influenza-like symptoms and a brief viremia (1-4 days), followed by an asymptomatic interval (Kaiser, 1999, 2008a; Lešničar et al., 1997). A second febrile stage starts around two to four weeks post-exposure, during which patients will develop one of four possible clinical manifestations: meningitis, meningo-encephalitis, meningo-encephalomyelitis or meningo-radiculoneuritis (Donoso Mantke et al., 2008a; Kaiser, 1999, 2008a).

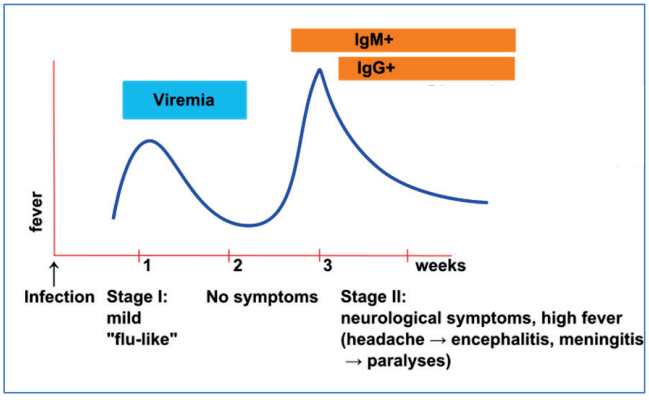

Figure I-7: Stages and symptoms of TBEV infection.
Adapted from RicHard-59 (2011).

This is when seroconversion starts developing and when usually medical advice is sought and the first diagnostic investigations take place (Holzmann, 2003). Natural infection results in lifelong immunity (Mickiene, 2008). A mortality rate of 0-3.9% has been reported in Europe (Donoso Mantke et al., 2008a; Haglund, 2002; Süss, 2008a), but morbidity and mortality rates increase with increasing age of the patient (Gunther et al., 1997; Lešničar et al., 1997; Mickiene et al., 2002).

In animals, neurological symptoms after TBEV-infection are often non-specific and can range from a (per)acute and lethal to a mild subacute course (Klaus et al., 2013). Typical canine TBE features pyrexia, lethargy/apathia, aggressiveness/fear, anorexia, and multifocal neurological signs (Bjöersdorff, 2002; Pfeffer and Dobler, 2011), including motor failures, paresis/paralysis and epileptic seizures (Bjöersdorff, 2002; Csángó et al., 2004; Janitza-Futterer, 2003; Kießling, 2005; Kirtz et al., 2001; Reiner and Fischer, 1998; Reiner et al., 1999; Stadtbaumer et al., 2004; Tipold et al., 1993; Wandeler et al., 1972; Weissenbock et al., 2010).

The early equine signs may resemble lameness or mild ataxia, but this quickly progresses to severe general signs with intermittent tonic-clonic convulsions and paresis/paralysis (Dietz and Huskamp, 2005; Grabner, 1993; Janitza-Futterer, 2003; Klaus et al., 2013; Long, 2011; Müller et al., 2006; Waldvogel et al., 1981). Similar clinical courses after natural/experimental infection have been observed in monkeys (Klaus et al., 2010a; Pripuzova et al., 2013; Süss et al., 2007), a mouflon (Bago et al., 2002) and even occasionally in the rodent reservoirs (Tonteri et al., 2013).

I.3.3 PROGNOSIS

Symptomatic patients need hospitalization for weeks to months, and between 35% and 58% of them are left with permanent sequelae called “post-encephalitic syndrome” (Gunther et al., 1997; Haglund et al., 1996; Kaiser, 1999, 2008a). These individuals require many years of costly treatment and rehabilitation due to long-term neurological symptoms, cognitive and psychiatric dysfunctions, social distress, inability to work and life quality loss. TBE comes at a very high cost to society and the health care system due to this high morbidity (Baumhackl, 2009; Kunz, 2008; Mickiene et al., 2002). The outcome of veterinary TBE is fatal much more often than humane cases and due to the very rapidly deteriorating clinical situation (within 3-7 days to a natural death), euthanasia is often requested by the owners (Pfeffer and Dobler, 2011; Waldvogel et al., 1981; Weissenbock et al., 2010).

I.3.4 Differential Diagnoses

Medical TBE has to be differentiated from many other infectious encephalitides, including Lyme borreliosis, human granulocytic anaplasmosis, herpes simplex viruses, varicella-zoster virus. Epstein-Barr virus, mumps virus, measles virus, and enteroviruses are considered to be the major causes of viral encephalitis in immunocompetent humans worldwide (Donoso Mantke et al., 2008b; Horger et al., 2012; Mickiene, 2008; Skarpaas et al., 2004; Storch, 2007). In Europe, the most important arboviral pathogens responsible for encephalitis are tick-borne encephalitis virus (TBEV), West Nile virus (WNV) and Sandfly fever virus (Donoso Mantke et al., 2008b; Kallio-Kokko et al., 2005).

Likewise, veterinary TBE has to be differentiated from e.g. Louping ill, rabies, Aujesky's disease, equine encephalitis viruses, distemper, West Nile virus, and metabolic or toxicologic causes (Bago et al., 2002; Dietz and Huskamp, 2005; Long, 2011; Müller et al., 2006; Smith, 2002). Additionally, it is known that *Ixodes* spp. may carry different pathogens at the same time, which they can also transmit to the human or animal host simultaneously, e.g. TBEV, *Borrelia burgdorferi* spp. (Lyme disease), *Anaplasma phagocytophilum* (Anaplasmosis), *Ehrlichia* spp. (medical: monocytic ehrlichiosis) and *Babesia* spp. (veterinary babesiosis)(Bajer et al., 2013; Kaiser, 2008a; Korenberg, 1994; Süss, 2011).

I.3.5 Treatment and Prevention

As opposed to Lyme borreliosis, which can be managed by antibiotic therapy, TBE can only be treated symptomatically through maintenance of hydration and caloric intake, analgesics/antipyretics and anticonvulsants/sedatives. Strict rest and physiotherapy are also paramount to avoid complications, both in humans and animals (Kirtz, 1999; Kunz, 2008; Kunze, 2008; Long, 2011; Pfeffer and Dobler, 2011; Tipold et al., 1993). Medical TBE can easily be prevented by vaccination, which is strongly advised for people of all ages who live or travel in endemic areas (Kunz, 2008; Mutz, 2008; Steffen, 2009). General prevention also includes avoidance of tick-infested areas, wearing light-colored clothing covering as much skin as possible, application of tick repellents, and skin inspection (BCFI, 2015; ITG, 2008; Luyasu, 2009; Mutz, 2008).

Veterinary prevention and landscape management may involve any or several of the following strategies:

1) Tick habitat avoidance for dogs (Dryden, 2009);
2) Use of spray or pour-on acaricides in domestic animals (Baggott et al., 2011; BCFI-vet, 2014; Berrada and Telford, 2009; Birkett et al., 2011; Dantas-Torres et al., 2012; Dryden, 2009; Jongejan et al., 2011; Jongejan et al., 2015; Müller et al., 2006; Otranto et al., 2010; Otranto and Wall, 2008; Taylor, 2012; Thein, 2009);
3) Use of acaricides in wild animals, by using passive application of a topical acaricide in deer poster bait stations or in a food bait or cotton wool box for rodents (Conover and Vail, 2015; Deblinger and Rimmer, 1991; Dolan et al., 2004; Gortazar et al., 2015; Grear et al., 2014; Leprince and Lane, 1996; Mejlon et al., 1995; Piesman, 2006; Poland, 2001; Pound et al., 2010; Stafford, 1991; Stafford et al., 2009);
4) Use of natural tick repellents, such as plant oils (Birkett et al., 2011; Jaenson et al., 2006; Otranto and Wall, 2008);
5) Pasture management, such as rotational grazing, keeping wildlife fenced out and keeping vegetation short, may help to reduce tick burden, if wildlife abundance is not too high (Dantas-Torres et al., 2012; Gortazar et al., 2015; Pound et al., 2010; Walker, 2011);
6) Inspection and tick removal (Dantas-Torres et al., 2012; Gortazar et al., 2015; Pound et al., 2010; Walker, 2011);
7) Wildlife population control (culling, hunting) (George, 1990; Gortazar et al., 2015)
8) Habitat management e.g. keeping vegetation and undergrowth short, acaricide spraying (CDC, 2014a; Dolan et al., 2004; Gortazar et al., 2014b; Piesman, 2006; Poland, 2001; Stafford, 2004; Tack et al., 2012; Uspensky, 1996; WHO, 2011);
9) Placement of tick traps with chemical or pheromone baits (Taylor, 2012; Otranto and Wall, 2008);
10) Off-license TBE vaccination (Klaus et al., 2011; Müller et al., 2006; Müller, 1997; Wurm et al., 2000);
11) Anti-tick vaccines, which remain in the research stage (de la Fuente and Kocan, 2003; de la Fuente et al., 2011; Guerrero et al., 2012; Jonsson et al., 2000; Merino et al., 2013; Odongo et al., 2007; Otranto and Wall, 2008; Schuijt et al., 2011; Trimnell et al., 2005; Willadsen et al., 2005), but seem to have a reducing effect on TBEV transmission (Labuda et al., 2006).

I.4 SPECIFIC DIAGNOSTIC ASSAYS

I.4.1 COMMERCIAL REAGENTS AND ASSAYS

An overview of specific commercial medical and veterinary reagents and assays currently available in Europe is shown in Table I-2 that were adapted from ENIVD (ENIVD, 2014).

Table I-2: Commercially available reagents and assays for TBEV diagnosis				
Name	**Type**	**Target**	**Contents / Matrices**	**Producer**
Serion® CFT Reagents TBE-virus	CFT Antigen Control Samples	Human	Antigen (Ag) – Control Ag - Positive Control - Negative Control	Institut Virion\Serion GmbH Serion Immundiagnostica GmbH http://www.virion-serion.de/en/
Serion® ELISA control TBE IgG/IgM	Control Samples	Human	/	Institut Virion\Serion GmbH Serion Immundiagnostica GmbH http://www.virion-serion.de/en/
Euroimmun® TBEV IgA/IgG/IgM IIFT	IFA	Human	IgG positive control IgM positive control Negative control IgA, IgG, IgM	Euroimmun http://www.euroimmun.de
Euroimmun® TBEV Controls	Control Samples	Human	Strain K23: IgM/IgG/for CSF/RF absorbent; Vienna antigen; IgM/IgG pairs of controls; IgG positive control	Euroimmun http://www.euroimmun.de
Test-Line® TBEV - CF-Ag Complement Fixation Antigen	CFT Antigen	Human	/	Testline http://www.testlinecd.com/
Virotech FSME/TBE IgG CSF Standards	Control samples	Human	Cerebrospinal fluid	Sekisui Diagnostics - Genzyme Virotech http://www.sekisuidiagnostics.com
Virotech FSME/TBE IgG Antibody Index Control	Control Samples	Human	Cerebrospinal fluid/serum pairs	Sekisui Diagnostics - Genzyme Virotech http://www.sekisuidiagnostics.com
Sacace® Tick-borne diseases (also TBEV) Real Time PCR kit	PCR	Human **Veterinary**	Biological materials	Sacace Biotechnologies http://www.sacace.com/
Genesig® TBE research qPCR kit	PCR and primer/probe mix	Human **Veterinary**	Biological materials	Genesig PrimerDesign Ltd http://www.genesig.com/
Reagena® ReaScan TBE IgM (Rapid Test)	LFD	Human	Serum - Cerebrospinal fluid	Oy Reagena Ltd http://www.reagena.fi/en/
Euroimmun® TBEV IIFT BIOCHIPs	IFA	Human	TBE virus infected and non-infected cells - single slide test	Euroimmun http://www.euroimmun.de
Western Blot	WB	Human **Veterinary**	Serum	Immuno http://www.progen.de/en/

Table I-2: Commercially available reagents and assays for TBEV diagnosis.
Adapted from European Network for Diagnostics of Imported Viral Diseases (ENIVD, 2014); CFT: complement fixation test; IFA: immunofluorescence Assay; LFD: lateral flow device; PCR: polymerase chain reaction; EIA: enzyme immune assay; ELISA: enzyme-linked immunosorbent assay; quant/qual: quantitative or qualitative interpretation

Table I-2: Commercially available reagents and assays for TBEV diagnosis (continued)				
Name	**Type**	**Target**	**Matrices**	**Producer**
Serion® ELISA classic FSME virus IgG/IgM quantitativ	ELISA quant/qual	Human	Serum – Plasma – Cerebrospinal fluid	Institut Virion\Serion GmbH Serion Immundiagnostica GmbH http://www.virion-serion.de/en/
Immunozym® FSME (TBE) IgG/IgM	ELISA quant/qual	Human	Serum – Plasma – Cerebrospinal fluid	Progen http://www.progen.de/en/
Immunozym® FSME IgG All species	ELISA quant/qual	**Veterinary**	Serum	Progen: http://www.progen.de/en/
Euroimmun® Anti-TBEV ELISA IgG/IgM	ELISA quant/qual	Human **Veterinary**	Serum – Plasma – Cerebrospinal fluid	Euroimmun http://www.euroimmun.de
Euroimmun® Anti-TBEV IFA or ELISA IgG/IgM	IFA ELISA quant/qual	**Veterinary**	Serum – Plasma – Cerebrospinal fluid	Euroimmun: http://www.euroimmun.de
Test-Line® EIA TBEV IgM or IgG+avidity	ELISA semiquant/ qual	Human	Serum – Plasma – Cerebrospinal fluid	Testline http://www.testlinecd.com/
Test-Line® EIA TBEV	ELISA semiquant /qual	Human **Veterinary**	Serum	Testline http://www.testlinecd.com/
Virotech® FSME IgG/IgM ELISA	ELISA quant/qual	Human	Serum – Cerebrospinal fluid (with extra controls)	Sekisui Diagnostics - Genzyme Virotech http://www.sekisuidiagnostics.com
NovaLisa™ TBEV IgM/IgG/IgG (plus)	ELISA quant/qual	Human	Serum – plasma (plus: quant. with extra control)	NovaTec Immundiagnostica GmbH http://www.novatec-id.com/
Reagena® TBE IgM and IgG EIA	ELISA quant/qual	Human	Serum	Oy Reagena Ltd http://www.reagena.fi/en/
IBL® TBE IgG or IgM ELISA	ELISA	Human	Serum – Plasma - Cerebrospinal fluid	IBL International GmbH http://www.ibl-international.com/en/tbe-fsme-igg
Abcam® TBE/FSME Virus plus IgG or IgG or IgG plus Human ELISA	ELISA Quant/qual	Human	Serum - Plasma	Abcam http://www.abcam.com/
Enzygnost® Anti-TBE/FSME Virus IgG/IgM in BEP-III-system	ELISA	Human **Veterinary**	Serum	Dade Behring Holding GmbH (Siemens) http://www.healthcare.siemens.com /infectious-disease-testing/systems/bep-iii-system/assays
Labor Alomed in-house ELISA	ELISA	**Veterinary**	Serum	Analytisches Labor Dr. Werner Müller Alomed http://www.alomed.de
DRG® TBE/FSME IgG EIA	ELISA	Human	Serum	DRG Diagnostics GmbH http://www.drg-diagnostics.de
Mastazyme FSME/ TBE IgM/IgG ELISA	ELISA	Human	Serum	Mast Group Diagnostica GmbH http://www.mast-diagnostica.com
RecomWell FSME / TBE Virus IgG/IgM	ELISA	Human	Serum – Plasma – Cerebrospinal fluid	Mikrogen Diagnostik GmbH http://www.mikrogen.de
ELISA-VIDITEST anti-TBEV IgM/IgG	ELISA	Human	Serum – Cerebrospinal fluid	VIDIA spol. s r.o. www.vidia.cz

The suitability of each type of test for specific TBE diagnosis depends on the stage in the biphasic pathogenic course of a TBEV infection as demonstrated in Figure I-8 (Holzmann, 2003). Like other arboviruses, TBE virus is only present in serum and cerebrospinal fluid (CSF) for 2-5 days during the first viraemic phase, hence **direct tests** have to be requested very timely (Donoso Mantke et al., 2007b; Holzmann, 2003; Mazlo and Szanto, 1978; Pfeffer and Dobler, 2011; Sonnenberg et al., 2004). These may include reverse transcription polymerase chain reaction (RT-PCR), virus isolation (VIS) and sequencing (Donoso Mantke et al., 2007a; Donoso Mantke et al., 2008a; ECDC, 2012; Holzmann, 2003; Kim et al., 2008; Saksida et al., 2005; Takashima et al., 1997), antigen-capture assays (Mikryukova et al., 2014; Ternovoi et al., 2007), immunohistochemistry (Donoso Mantke et al., 2007a; Holzmann, 2003; Süss et al., 2007; Tipold et al., 1993; Weissenbock et al., 2010) and electron microscopy (Mazlo and Szanto, 1978).

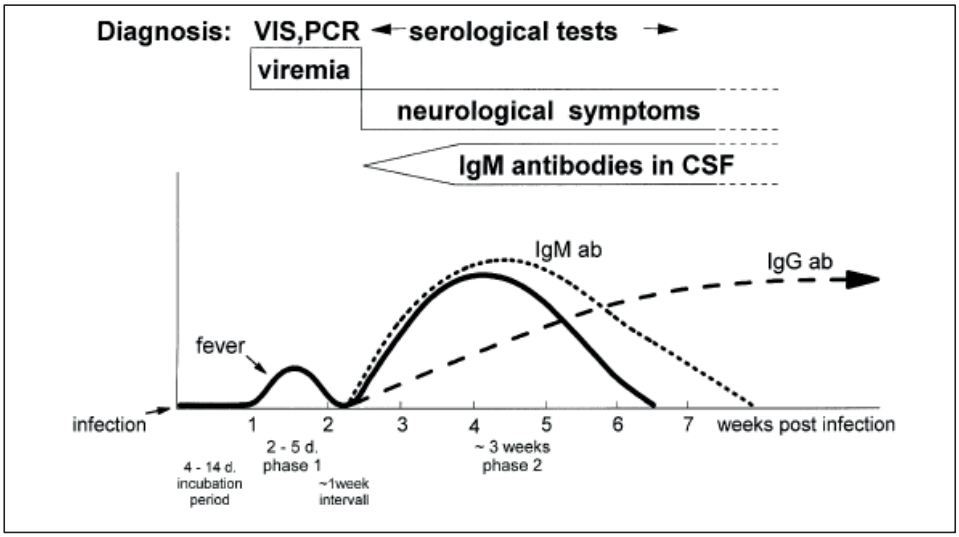

Figure I-8: Suitable tests for specific TBE diagnosis.
According to biphasic course of a TBEV infection with symptoms and antibody development (source: Holzmann, 2003); PCR: polymerase chain reaction; VIS: virus isolation; IgM ab - IgG ab: immunoglobulins of class M or G.

Sensitive **real-time PCR** methods are often used in veterinary and vector TBE surveillance and in clinical diagnostics (Achazi et al., 2011; Andreassen et al., 2012; Huang et al., 2001; Puchhammer-Stockl et al., 1995; Schwaiger and Cassinotti, 2003; Wójcik-Fatla et al., 2011); primers and probes were listed (Achazi et al., 2011; Gaumann et al., 2010; Katargina et al., 2013). Subsequent multiplexing and sequencing of positive samples is strongly advised for improved FE-S-W-TBEV subtype differentiation and to study TBEV molecular-epidemiology. This is based on primer sets for the well studied TBEV strains: Western (Hypr, Noedorfl), Far Eastern (Sofjin), Siberian (Est54) as well as (Ruzek et al., 2007b).

A number of **animal models** may be employed to study the neuropathogenicity of TBEV (Mansfield et al., 2009) or for anti-tick vaccination trials (Labuda et al., 2006; Trimnell et al., 2005). This includes mice (Andzhaparidze et al., 1978; Denk and Kovac, 1966; Labuda et al., 2006; Mandl, 2005; Trimnell et al., 2005), hamsters (Andzhaparidze et al., 1978) and monkeys (Andzhaparidze et al., 1978; Pripuzova et al., 2013; Zlontnik et al., 1976).

Antibodies are almost always present in serum and CSF at the time of CNS symptoms (≥2-4 weeks after the tick bite, second phase) and so the diagnosis of most TBE cases/exposures will be a serological one (Donoso Mantke et al., 2008a; Holzmann, 2003; Sonnenberg et al., 2004). In parallel with the pathogenesis of a TBEV infection, **IgM** based tests allow a qualitative interpretation of an acute clinical situation while **IgG** serves as a more quantitative and long term seroconversion indicator. IgM-antibodies may be detectable in serum for several months after infection, whereas IgG-antibodies may persist for a lifetime and prevent reinfection (Holzmann, 2003; Mickiene, 2008; Remoli et al., 2015).

Currently, the following types of **medical serology** are used for humans in EU countries: enzyme-linked immunosorbent assays (ELISA), complement fixation tests (CFT), virus seroneutralisation or plaque reduction neutralisation tests (SNT/PRNT), haemagglutination inhibition assays (HIT), immunofluorescence assays (IFAT), Western blots (WB) and Luminex antibody tests (LAT) (ECDC, 2012; ENIVD, 2014), with well-accepted standardized methods (Clarke and Casals, 1958; Heinz and Kunz, 1975; Shope and Sather, 1979; Stage, 1992; Treib et al., 1996). In ELISA, samples with VIEU units ≥127/ml are considered positive (Holzmann et al., 1996; Litzba et al., 2014).

In comparison, relatively few serological tests are commercially available for **veterinary use**: indirect immunofluorescence assay (Euroimmun®), three ELISA kits (Progen® – Testline® - Enzygnost®), or Western Blot (Immuno®). The ELISA manufacturers claim that the kits are be suitable for "all species" (Progen, 2014; Progen, 2006) or "all vertebrates except mice" (Testline, 2015) or not mentioned (Siemens, 2016). To our knowledge, veterinary test evaluation studies have not been published for any of these kits, except for the Progen® kit (Klaus et al., 2011).

For an unequivocal diagnosis of acute disease, **both IgM and IgG** should be tested and should be positive (Donoso Mantke et al., 2008a; Holzmann, 2003; RKI, 2009). **Paired sera** are strongly indicated to demonstrate a clear rise in IgG antibody titers (Domingo et al., 2012; Donoso Mantke et al., 2008a; Holzmann, 2003).

In dogs and horses, only a detection of IgM or a four-fold rise in IgG antibodies in paired serum (or liquor) 2-3 weeks apart confirms the diagnosis (Müller et al., 2006; Pfeffer and Dobler, 2011). When the asymptomatic seroprevalence is expected to be high in known endemic areas, it is even more important to test paired sera, since in these cases antibody presence does not necessarily imply causality (Müller et al., 2006). Since DIVA-tests to "Distinguish Infected from Vaccinated Animals" are not available, flaviviral vaccination always needs to be ruled out in the anamnesis (Thein, 2009).

A major issue when using serology (especially IgG assays) for TBE diagnosis is the **frequent cross-reactions** induced by other flavivirus infections or vaccinations. In humans it most often concerns Yellow Fever virus (YFV), Dengue virus (DEV), West Nile virus (WNV) and Japanese Encephalitis virus (JEV), and Louping ill virus (LIV) (Aslan Basbulut et al., 2012; Beck et al., 2013; Domingo et al., 2012; Donoso Mantke et al., 2008a; Holzmann, 2003; Niedrig et al., 2007a; Niedrig et al., 2007b).

In the case of European dogs, exposure to WNV (though dogs are not easily infected) or LIV (which produces similar canine encephalitis cases) may interfere (Dobler, 2010; Klimeš et al., 2001; Müller, 2010; Pfeffer and Dobler, 2011). For **horses,** TBEV-, WNV-, DENV- and -SLEV-seropositivity interfere with WNV-ELISA and WNV-SNT (Ledermann et al., 2011; Rushton et al., 2013) and in the case of ruminants and for pigs/wild boar, WNV/LIV or WNV/LIV/JEV are good candidates to rule out respectively (Hubalek et al., 2014).

It is strongly recommended to check for flaviviral cross-reactivity by verifying any TBE-positive results from screening test. Hereto, the use of comparative and specific in-house **seroneutralisation** tests (plaque reduction or microneutralisation tests) are preferred, despite the need for specialized biosafety level 2+ or 3 facilities and higher costs (Achazi et al., 2011; Donoso Mantke et al., 2008a; Holzmann, 2003; Holzmann et al., 1996; Janitza-Futterer, 2003; Kiffner et al., 2012; Klaus et al., 2011; Klaus et al., 2014; Litzba et al., 2014; Sîkutova et al., 2009; Vene et al., 1998; Venturi et al., 2009; Weissbach and Hirsch, 2015).

TBE-SNT titers from ≥1/10 are considered positive and offer sufficient protection in humans (Holzmann et al., 1996; Kollaritsch et al., 2011a; Venturi et al., 2006; Weissbach and Hirsch, 2015; WHO, 2011) and when titers in a particular SNT/IFAT are ≥4-fold higher against TBEV than against other flaviviruses, this is accepted as a proof of specificity (Escribano-Romero et al., 2015; Litzba et al., 2014).

I.4.2 Quality Assessment of TBEV Diagnostic Assays

In RT-PCR proficiency tests, the (Schwaiger and Cassinotti, 2003) **RT-PCR** protocol used with commercial extraction kits usually leads to significantly improved classification. This protocol has an analytical sensitivity ≥10 copies of TBEV synthetic transcript in presence of 50 copies of internal control. In the proficiency test by Donoso Mantke (2007), a model was used to show the 50% of all test results would be correctly positive with 80 copies of virus RNA/ml of sample, and 95% with more than 350,000 copies/ml (Donoso Mantke et al., 2007a). Additionally, quantitative RT-PCR has offered improved rapidity, sensitivity, reproducibility and reduced risk of cross-contamination (Donoso Mantke et al., 2007b). Currently, there is still a need to improve the sensitivity of RT-PCR (Achazi et al., 2011).

When older in-house and commercial **serological tests** were evaluated for routine medical use, the diagnostic accuracy (sample classification potential) was often quite low and variable: diagnostic sensitivity (DSe) 73-99% and diagnostic specificity (DSp) 14-94%, with many flaviviral cross-reactions (Holzmann et al., 1996; Niedrig et al., 2001; Sonnenberg et al., 2004).

Since then, commercial flaviviral tests have much improved accuracy in proficiency tests. In 2007, overall European laboratory accuracy was between 58-96%, IgM/IgG seropositives were correctly recognized by 46-88% and 83-95% of the laboratories respectively. False IgG positive results were obtained with DENV, WNV and negative sera (Niedrig et al., 2007a).

Hence, cross-reactions and unspecific reactions can still not be excluded 100%, especially in IgG ELISA but even in the gold standard SNT (Domingo et al., 2012; Litzba et al., 2014; Niedrig et al., 2007a; Niedrig et al., 2007b; Weissbach and Hirsch, 2015). Currently, reliable SNT protocols are now used in most reference laboratories and IFA also is a good alternative for doubtful IgG ELISA results. It is advised that commercial ELISA companies should still continue to standardize the existing kits (VIEU units, standard samples, cut-offs) (Litzba et al., 2014). When executed by expert laboratories under external quality assurance programs, and used as a confirmatory test with well-characterized controls and standardized protocols, SNT strongly improves the quality of TBE diagnosis (Donoso Mantke et al., 2007a; Donoso Mantke et al., 2008a; Niedrig et al., 2007a).

Since the available commercial veterinary serological assays are not fully **validated** for all relevant **veterinary species**, it is important to include **control sera** for the correct species (Müller et al., 2006). Klaus et al. evaluated the Immunozym® FSME IgM in an adapted IgG+IgM protocol (Klaus et al., 2011; Müller, 1997) and the All-species IgG ELISA kits for goats, versus an adapted SNT test as veterinary gold standard (Holzmann et al., 1996; Klaus et al., 2010c). The All species kit (IgG) showed a DSe and DSp of 57% and100%; for the medical ELISA kit the DSe and DSp were 89% and 95% respectively. Species-specific veterinary unit cut-offs were afterwards calculated for horses, cattle, sheep, goats, pigs, mice, dogs, rabbits and monkeys, using field sera from assumed negative populations and vaccinated animals (Klaus et al., 2011).

The study also confirmed very high **sensitivity of SNT** on the vaccinated samples with a satisfactory correlation of the observed results between the ELISA-kits and the SNT. In comparison to serum ELISA, **milk** ELISA in cattle with the slightly modified All Species IgG ELISA reached a diagnostic specificity (DSp) of 96.4%, a positive predictive value (PPV) of 37.5% and a diagnostic sensitivity (DSe) of merely 25% (Leutloff et al., 2006).

I.5 TBE EPIDEMIOLOCIAL SURVEILLANCE

I.5.1 MEDICAL SURVEILLANCE

Sero-epidemiological studies in occupational TBE risk groups (e.g. foresters, hunters, farmers, military personnel) or in the overall unvaccinated population proved useful in the early days of TBE surveillance, with seroprevalences of 0-48% recorded, even in areas where no clinical cases were officially reported (Asmera and Heinz, 1972; Drăgănescu et al., 1975; Günes et al., 2010; Körting, 1981; Matile et al., 1981; Oehme et al., 2002; Walder et al., 2006; Wohlfarth et al., 2009). However, from the nineties onwards professionals were often widely vaccinated as soon as endemic foci were detected (Kunz, 1992; Roggendorf et al., 1994), hence it became difficult to perform meaningful serosurveillance in humans (Gerth et al., 1995; Kunz, 2003; Kunze, 2011).

It is vital to (inter)nationally **report clinical TBE cases** to implement public health interventions, for detection/description of endemic foci and for risk assessments (ECDC, 2012; Kollaritsch et al., 2011a; Kollaritsch et al., 2011b; Süss, 2011; WHO, 2011). Although by 2010 comprehensive national surveillance systems with mandatory reporting were already implemented for TBE in the majority of EU countries (in 60% of MS), important differences still existed in terms of case definitions, clinical syndromes reported (in 50% of MS), and laboratory testing protocols (ECDC, 2012). Since September 2012, TBE is included on the EU notifiable list of human diseases, with an official medical case definition based on the clinical picture of TBE (biphasic: flu-like + meningo-encephalitis), the serological diagnosis (IgM/IgG, cross-reactions), and on epidemiological factors (tick exposure, raw milk consumption, risk areas, vaccination status)(Amato-Gauci and Zeller, 2012; Süss, 2011; Süss et al., 2010).

Human incidence is currently usually reported at the country level as in Figures I-9 and I-10 (Beck et al., 2013; ECDC, 2012). However, for TBE risk assessment, for distinguishing real incidence trends, as well as for the development of a multi-factorial predictive framework, case data should ideally be collected at higher resolution to take into account the very variable biotic and abiotic drivers of TBE disease risk (ECDC, 2012; Randolph and Sumilo, 2007). The medical incidence recorded during 60 years of surveillance has shown considerable spatio-temporal heterogeneity between and within countries (Mansfield et al., 2009; Randolph, 2008; Randolph and Sumilo, 2007; Süss, 2011).

However, a 400% increase in TBE morbidity was observed in central Europe during the 1990's (Süss, 2008b), and the virus can now be found in regions that were previously unaffected (Charrel et al., 2004). Shifting distributions and patchiness have also been observed, mainly in Western Europe (Jääskeläinen et al., 2006).

The number of **alimentary** (milk-borne) TBE cases has increased in many endemic areas (Balogh et al., 2010; Holzmann et al., 2009; Kerbo et al., 2005; Kriz et al., 2009; Kriz et al., 2014; Labuda et al., 2002; Leutloff et al., 2006) and currently accounts for approximately 1-30% of the total incidence depending on the year and the country (Süss, 2011). Alimentary TBE usually manifests as small to large point outbreaks (Hubalek et al., 1986; Labuda et al., 2002; Süss, 2011), and can occasionally be traceable to a single or a few ruminants (Balogh et al., 2010; Caini et al., 2012; Hudopisk et al., 2013).

Medical TBE case-reporting tends to be **unreliable** even in regions where TBE is highly endemic (Süss, 2008b, 2011), as incidence is often based on place of residence – not necessarily place of infection, since seasonal data and the necessary local spatial resolution are often missing (ECDC, 2012), and as incompatibility exists with the prevalence of TBEV in ticks (Bormane et al., 2004; Brinkley et al., 2008; Makowka et al., 2009; Süss et al., 2006). Additionally, it has been amply shown that the majority of human TBEV exposures do not lead to clinical signs, hence the confirmed cases represent only the very tip of the zoonotic iceberg (Drelich et al., 2014; Randolph and Sumilo, 2007).

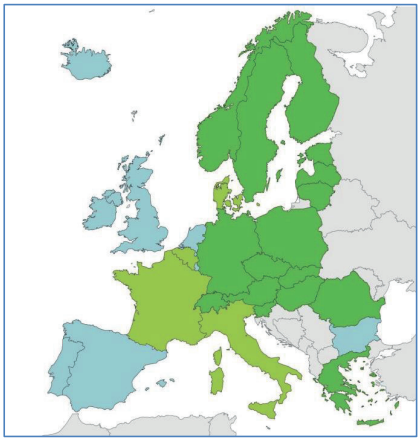

Figure I-9: Overview of TBE surveillance implemented in EU/EFTA countries.
Dark green: mandatory surveillance, light green: other surveillance, blue: no surveillance; extract from ECDC, 2012.

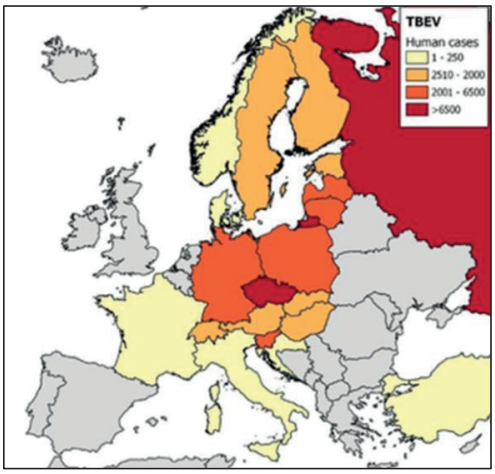

Figure I-10: Map of reported human TBE cases in Europe.
Aggregated human cases (2000–2009) per country; Beck et al., 2013

Hence, the current medical data do not really reflect the complete picture or the complexity of TBE's epidemiology correctly and can severely distort or underestimate the true TBEV prevalence in an endemic focus (Drelich et al., 2014; ECDC, 2012; Randolph and Sumilo, 2007). As a consequence, ECDC recommends the additional collection of data from tick and animal surveys to better define TBE endemic areas (ECDC, 2012). Not only are people mobile, but so are the hosts that may transport virus-infected engorged ticks to other parts of a given habitat (Waldenström et al., 2007).

When endemic foci shift and (re-)emerge spatially and temporally they may be discovered and characterized by virus detection in ticks and serosurveillance in domestic and wildlife sentinel animals, and this in presence or absence of any autochthonous human cases (ECDC, 2012; Gaumann et al., 2010; Kollaritsch et al., 2011b; Labuda et al., 2002; Leutloff et al., 2006; Rieger et al., 1997; Rieger et al., 1999; Süss, 2011; WHO, 2011).

I.5.2 VETERINARY SURVEILLANCE

I.5.2.1 Domestic Species

a. Companion Animal Studies

Dogs are frequently exposed to ticks (Beck et al., 2014; Claerebout et al., 2013; Foldvari and Farkas, 2005; Schreiber et al., 2014) and may experience asymptomatic TBEV-infections which raise a clearly detectable long-lasting immune response to the virus (Pfeffer and Dobler, 2011). Clincal canine TBE cases have been known in endemic areas for over 40 years and seem to be emerging among Europe's canine population (Beugnet and Marie, 2009; Leschnik et al., 2002) with its distribution expanding over Western Europe in parallel with human TBE spread.

Until now, only small numbers of canine clinical cases were published, often in local language case reports from known endemic foci of Sweden (Åblad, 2007; Björsdorff, 2002; James, 2008; Lindblad, 1960; Wandeler et al., 1972), Switzerland (Gresikova et al., 1972; Tipold et al., 1993; Wandeler et al., 1972), Austria (Kirtz, 1999; Weissenbock et al., 2010), Germany (Reiner and Fischer, 1998), and Italy (Zanoni et al., 2009), and summarized in (Pfeffer and Dobler, 2011).

Several **canine antibody surveys** have been performed in endemic areas in the Northern hemisphere (see Table I-3). In the majority of these canine subjects no CNS clinic was observed and seroprevalence was estimated between <2% and 31% with ELISA or HIT. When the results are confirmed by SNT testing, the seroprevalence turns out lower than estimated by the screening test (Kirtz, 1999; Lindhe et al., 2009; Pfeffer and Dobler, 2011).

More asymptomatic and clinical caninecases are likely to be detected as awareness increases in the veterinary community. (Beugnet and Marie, 2009; Leschnik et al., 2002; Pfeffer and Dobler, 2011). Furthermore, dogs live close to their human owners and may form a human exposure risk. This species may also travel which can sometimes obscure the exact place and time of TBEV-exposure, as in humans (Beugnet and Marie, 2009; Leschnik et al., 2002; Pfeffer and Dobler, 2011) Pfeffer and Dobler, 2013).

Cats have not been used in any TBE sentinel study so far. However, they are more sedentary than dogs and may predate TBEV-reservoirs. Most cats stay within 300 meters of structures with a home range around 0.05-3 km^2 (Gehrt et al., 2013; Horn et al., 2011; Kitts-Morgan et al., 2015). They are documented tick hosts and may carry *I. ricinus* and pathogens into human habitats (Chomel, 2013; Claerebout et al., 2013; Jongejan and Uilenberg, 2013; Pfeffer and Dobler, 2013). Until present, cats have not been reported to develop clinical TBE (Greene, 2013; Leschnik et al., 2002; Shaw, 2005).

The proportion of asymptomatic **equine** infections is not exactly known, but seroprevalence studies have always found many asymptomatic seropositives (Janitza-Futterer, 2003; Luckschander, 1998; Rushton et al., 2013; Vesenjak-Hirjan et al., 1976a, b, c). **Equine cases** have been described (Grabner, 1993; Luckschander et al., 1999; Waldvogel et al., 1981).

Sentinel seroprevalence studies in **horses** (Table I-3) were performed in former Yugoslavia (Vesenjak-Hirjan et al., 1976a, b, c) and in Hungary (Sîkutova et al., 2009), and in Austria, where increasing prevalences of 14% to 26.1% ELISA-positives were detected, in line with human TBE case incidence, and most seropositive horses originating from known endemic areas (Janitza-Futterer, 2003; Luckschander et al., 1999; Müller et al., 2006; Rushton et al., 2013). Horses are suitable for mapping exercises and to detect and investigate endemic risk areas, since they are relatively less mobile than dogs (Imhoff et al., 2015; Janitza-Futterer, 2003; Klaus et al., 2013).

b. Domestic Ruminant Studies

Though **ruminants** are mostly infected with TBEV asymptomatically, they are good **sentinels** in endemic areas (Bjöersdorff, 2002; Leschnik et al., 2002; Sîkutova et al., 2009) Cattle seroprevalence in known TBEV-endemic foci may be low around 0-4%, but can be much higher, from 13-30% and up to 91% in certain high risk areas (Table I-3)(Cisak et al., 2010). There may be remarkable differences in within-herd prevalences (Gerth et al., 1995) and seroprevalence fluctuations throughout the seasons (Ernek and Kozuch, 1970; Rieger et al., 1997), which reflect the temporo-spatial nature of TBE risk well (Imhoff et al., 2015).

In raw milk of high risk areas, RNA and antibody prevalences of 11-22% and 0-15% respectively were found (Cisak et al., 2010). TBEV transmission by raw milk presents a potential food chain risk (Verraes et al., 2015). In case of alimentary TBE outbreaks, TBEV RNA may sometimes be detected in serum and milk of the source animal(s), if they are still in the viraemic phase (Caini et al., 2012; Hudopisk et al., 2013).

Table I-3: Overview of veterinary sentinel studies in domestic animals				
Species	***Country***	***Test***	***Prevalence and sample size***	***References***
Dogs	**Belgium**	**ELISA - SNT**	**1.13% - 0.11% (n=880)**	**(Roelandt et al., 2011)**
Canis familiaris	Austria	Serology	24% (n=545)	(Kirtz, 1999)
		HIT	22.22% (n=36)	(Sixl et al., 1973)
	Germany	EIA	0-31% (n=100)	(Müller, 1997)
		EIA	< 2-31% ~region (n=1,000)	(Müller, 2000)
		ELISA	31.38% (n= 54 healthy) 53.57% (n= 56 CNS)	(Reiner et al., 2002)
		ELISA	29.2% (n=243)	(Janitza-Futterer, 2003)
	Sweden	Serology	7.06% (n=255)	(Wattle, 1992)
	Switzerland	Serology	3.6% (n=657)	(Matile et al., 1981)
	Japan	SNT - Isolation	73.68% (n=19) 30% (n=10)	(Takashima et al., 1997)
	Czech Republic	HIT	3.3% (n=151)	(Klimeš et al., 2001)
	Norway	ELISA	16.4% (n=317)	(Csángó et al., 2004)
	Denmark	ELISA/SNT	30% / 4.8% (n=125)	(Lindhe et al., 2009)
	Netherlands	ELISA/SNT/HI	0.0% (n=24)	(van der Poel et al., 2005)
Horses	Germany	ELISA	23.4% (n=205)	(Janitza-Futterer, 2003)
Equus caballus		ELISA	5.2% (n=155)	(Klaus et al., 2013)
	Croatia	HIT/CFT/SNT	91.67%/100%/86.96% (n=24)	(Vesenjak-Hirjan et al., 1976b)
		HIT/CFT/SNT	62.50%/45.83%/58.83% (n=24)	(Vesenjak-Hirjan et al., 1976c)
	Austria	ELISA/SNT	35.61% / 12.79% (n=469)	(Luckschander, 1998; Luckschander et al., 1999)
		ELISA/SNT	2.9% (n=240)	(Müller et al., 2006)
		ELISA/SNT	26.1% (n=257)	(Rushton et al., 2013)
	Hungary	ELISA/SNT	0.0% (n=40)	(Sîkutova et al., 2009)
Ruminant Milk	Poland Germany	RT-PCR ELISA	22.2%-14.8% (n=27 sheep); 20.7%-0% (n= 29 goats); 11.1%-3.2% (n=63 cows)	(Cisak et al., 2010)
		ELISA	1-8% (n=506 cows)	(Leutloff et al., 2006)
Pigs	Hungary	HIT	0% (n=217)	(Girjabu et al., 1985)
Sus scrofa	Austria	HIT / SNT	4 pigs exposed to goat milk	(Holzmann et al., 2009)
		HIT	0.41% (n=244)	(Sixl et al., 1973)
Birds	Hungary	HIT	0% (n=214 geese + 171 ducks)	(Girjabu et al., 1985)

Table I-3 (Cont.): Overview of veterinary sentinel studies in domestic animals				
Species	*Country*	*Test*	*Prevalence and sample size*	*References*
Cattle *Bos taurus*	**Belgium**	**ELISA - SNT**	**3.85% - 2.61% pos and 0.9% borderline (n=650)**	**(Roelandt et al., 2014)**
	Slovakia	SNT	Spring 0-3.1%; Autumn: 7.3-60% (n=340)	(Ernek and Kozuch, 1970)
		HIT - SNT	13% (n=60)	(Hubalek et al., 1986)
	Finland	Serology	91% (n=?)	(Brummer-Korvenkontio et al., 1973)
	Austria	HIT	10.64% (n=94)	(Sixl et al., 1973)
	Croatia	HIT - CFT - SNT	51.16%/16.33%/53.73% (n=86)	(Vesenjak-Hirjan et al., 1976b)
		HIT - CFT -SNT	54.54%/1.82%/60.00% (n=55)	(Vesenjak-Hirjan et al., 1976c)
	Germany	ELISA	2-10%~region (n=506)	(Leutloff et al., 2006)
	Poland	ELISA	4.1% (n=123)	(Cisak et al., 2012)
	Germany	ELISA	6.3-15.0% (n=126 before and after grazing)	(Rieger et al., 1997)
	Lithuania	HIT	2.4% (n=423)	(Juceviciene et al., 2005)
	Russia	HIT - CFT	17% - 29% (n=1,414)	(Korenberg et al., 1984)
	Japan	ELISA - SNT	0% (n=54)	(Takashima et al., 1997)
	Hungary	HIT	1.4% (n=214)	(Girjabu et al., 1985)
		ELISA - SNT	26.5% (n= 260)	(Sîkutova et al., 2009)
	Netherlands	ELISA - SNT - HIT	4.44% - 0.0% - 0.0% (n=180)	(van der Poel et al., 2005)
Small ruminants *Ovis aries* *Capra hirus*	Hungary	HIT	1.1% (n= 179 goats) ; 0% (n= 161 sheep)	(Girjabu et al., 1985)
		ELISA - SNT	7.0% (n=100 sheep)	(Sîkutova et al., 2009)
	Switzerland	ELISA - homemade absorption test	4.3% / 1.2% (n=4,114 goats)	(Rieille et al., 2013)
	Italy	Serology	12.0% (n=459 goats)	(Rizzoli et al., 2007)
	Germany	ELISA - SNT	1/7 goats positive in non-endemic area	(Klaus et al., 2010c)
		ELISA - SNT	9% (n=100)	(Klaus et al., 2010a)
		ELISA - SNT	3.2% (n=3,793 goats) 5.9% (n= 3,590 sheep)	(Klaus et al., 2012)
		ELISA - SNT	7.0% (n= 701 goats)	(Klaus et al., 2014)
	Lithuania	HIT	4.2% (n=118 sheep); 0.7% (n=561 goats)	(Juceviciene et al., 2005)
	Russia	HIT - SNT - CFT	27% - 25% - 32% (n=1,641 goats)	(Korenberg et al., 1984)
	Slovakia	HIT	3.29% (n=700 sheep) ; 37.12% (n=167 goats)	(Labuda et al., 2002)
		HIT - SNT	18% (n=120 sheep); 54% (n=26 goats)	(Hubalek et al., 1986)
	Croatia	HIT	56.09% (n=82 sheep)	(Vesenjak-Hirjan et al., 1976a)

I.5.2.2 Wildlife Species

a. Requirements for Surveillance

Generally, wildlife disease surveillance is most useful and informative when it is risk-based, targeted, sentinel or syndromic surveillance (Warns-Petit et al., 2009), although the latter would not be applicable to TBEV (asymptomatic infections). The third study in this thesis was performed in wild boar (Chapter V) and confirmed a potentially important role for **wildlife sentinel** surveillance in TBE risk assessment, mapping, control and prevention of TBE.

An important problem in epidemiological wildlife research is the estimation of the **population's size** (abundance, density) and **demographic structure** (age groups, sexes). This is complicated since often there are no gold standard methods, as e.g. for roe deer and wild boar (Bonenfant and Gaillard, 2015; Gortazar et al., 2014a). Therefore, a good cooperation between veterinarians and wildlife managers is necessary (Ryser-Degiorgis, 2013; Warns-Petit et al., 2009), Fortunately, international efforts to harmonize counting methods are now initiated and ongoing, e.g. in the APHAEA project www.aphaea.eu (Ryser-Degiorgis, 2013).

For **large game species, hunting bag** data are currently the only available index that could be harmonized at National/European scale, if the hunting effort (animals-per-hunter-day) is documented in detail and is not limited by quota. Moreover, larger sampling areas (region/country scale) must be considered to reduce bias (Acevedo et al., 2014; Apollonio et al., 2010; Engeman et al., 2013; Gortazar et al., 2014a; Imperio et al., 2010). The hunting bag data can be used with spatially explicit modelling procedures to obtain distribution and abundance (Acevedo et al., 2014; Bosch et al., 2012). Such spatial models have also been used with small sample sizes to give accurate density estimates e.g. of extensively kept livestock (Bryssinckx et al., 2014; Bryssinckx et al., 2012).

For small **songbirds** and **lagomorphs** point counts or line transects are to be preferred for density and distribution estimations (Acevedo and Gortazar, 2014; Barrio et al., 2010; Buckland et al., 2004; Fernández-de-Simón et al., 2011; Gortázar et al., 2015; Gregory et al., 2004; Thomas et al., 2010). Ornithological databases on migrating or resident ringed birds are also key resources (Gortázar et al., 2015; Martínez et al., 2009). In **voles and mice**, the gold standard is live trapping with a capture-mark-recapture method (Southwood and Henderson, 2000) (Drewes et al., 2015; Sibbald et al., 2006), or the less laborious lethal snap trapping under specific permits. Both methods are known to give well correlated estimates (Drewes et al., 2015; Hanski et al., 1994).

Sampling may be called convenience/opportunistic at low sample sizes, as one simply uses everything that is collected over several years (Boadella et al., 2011; Ryser-Degiorgis, 2013; Tavernier et al., 2015). This is an acceptable method, as long as the sampling stays representative of the population (spatial/demographics/habitat preferences) {Leutloff, 2006 #1106; (Roelandt et al., 2016). More ideally, sampling should be probability-based, randomized and stratified (Boadella et al., 2011; Leutloff et al., 2006; OIE, 2010; Ryser-Degiorgis, 2013). Sufficient care should be taken in the study design to establish a reasonable sampling effort and sample size, and to properly stratify the population according to relevant infection risk factors (Boadella et al., 2011; Munoz et al., 2010; Ryser-Degiorgis, 2013; Vicente et al., 2007; Vicente et al., 2006).

An absolute necessity for wildlife surveillance is to dispose of sample and data **storage facilities** to provide centralized baseline data. Furthermore, this will enable multi-disciplinary retrospective, longitudinal, prospective or trend analyses of emerging infectious diseases such as Bluetongue and Schmallenberg, provided that the samples are stored long term (Boadella et al., 2011; Linden et al., 2010; Müller, 1997; Ryser-Degiorgis, 2013; Warns-Petit et al., 2009). Regional and local 24-hour freezer networks and laboratories are often used (AEP, 2014; Lamarque and Artois, 1997; Linden, 2005). Alternatively, a centralized delivery and storage location can be used together with clear shipping and handling instructions for hunters and members of the public (ANB, 2015; CWHC, 2015; MEDI, 2015).

b. Wildlife TBEV Studies

Table I-4 shows an overview of wild species that have been used as sentinels (Tables I-4). **Game species** have been used frequently as sentinels for TBE(V) surveillance and for marking out risk areas, as flavivirus seroprevalence is usually higher and therefore presence is easier to detect than in domestic animals (Boadella et al., 2012; Jimenez-Clavero et al., 2007). These seroprevalences usually fall within medium (2.5-15%) to high (25-50%) intervals (Gerth et al., 1995; Kiffner et al., 2012) and seem to be representative of human incidence and of the risk in the area (Cisak et al., 2010; Gerth et al., 1995; Rieger et al., 1999). Authors' opinions are divided as to which game species may be the "best" sentinel. However, this may be determined by local ecological factors, such as relative population densities and habitat availability.

Cervids play an important role in TBE surveillance and positive correlations have been made between the abundance of **roe deer**, clusters of TBE seroprevalence and TBE risk (Carpi et al., 2009; Carpi et al., 2008; Gerth et al., 1995; Rizzoli et al., 2014). Their home ranges are approximately 0.5-1 km^2 with occasional dispersal up to 10-20km (Duscher et al., 2015a; Gerth et al., 1995; Radda et al., 1968; Reimoser et al., 1999). The roe deer population is well spread over most of the Belgian territory and roe deer hunting bags have increased steadily between 1960-2012 (Casaer and Licoppe, 2010; Scheppers et al., 2013). Free-living **Red deer** are currently present in southern Belgium (DGARNE, 2015a; Mercelis, 2003; Prévot and Licoppe, 2013; Scheppers et al., 2013, 2014).

Though traditionally **deer** spp. (Table I-4) have been considered as the main amplification host for *I. ricinus* ticks (Borcic et al., 1990; Skarphedinsson et al., 2005) (Gómez-Martínez, 2014; Kiffner et al., 2011; Kiffner et al., 2012; Knap and Avsic-Zupanc, 2013; Rizzoli et al., 2009) and deer hunting bag data may correlate well with TBE incidence (Kiffner et al., 2010; Knap and Avsic-Zupanc, 2013; Rizzoli et al., 2009; Zeman and Januska, 1999), it has been recently shown that large or increasing, endemic or reintroduced populations of **wild boar** may play an equal or larger epidemiological role in comparison to cattle and deer: see Table I-4 (Cisak et al., 2012; Gómez-Martínez, 2014; Kriz et al., 2014). Unregulated wildlife populations showing rapid exponential growth, such as the current European wild boar populations, may partially drive the spread of TBEV into suburban areas (Kriz et al., 2014).

Red Foxes are highly exposed to *I. rixinus* ticks of all life stages (Meyer-Kayser et al., 2012) in their habitats. They have a home range of approx. 7-10 km^2 and play an epidemiological role in urban TBE. The seroprevalence is often, but not always well correlated with TBE-risk (Haemig et al., 2011; Palo, 2014; Rizzoli et al., 2014): the reason for this is unknown. Nonetheless, foxes should not be disregarded as TBE-sentinels at the community level, since the species is present all over Belgium (DGARNE, 2015b; Libois, 2006; Van Den Berge and Pauw, 2003). In Flanders, the red fox has been recolonizing areas during the last decades of the 20^{th} century, after a long absence (DGARNE, 2015b; Van Herzele et al., 2015).

Belgian wild boar populations are increasing in density and spreading and as a result may become an important tick/TBE risk factor (Apollonio et al., 2010; Rizzoli et al., 2014). During the 20^{th} century, wild boar populations remained in Wallonia (Casaer and Licoppe, 2010; Scheppers et al., 2013),

expanding there over the last 30 years while progressively invading all agro-forested areas (DGARNE, 2015c; Prévot and Licoppe, 2013; Prévot and Morelle, 2012). More recently, a Flemish wild boar population has been developing north of Samber and Maas rivers in northern Belgium. This population is now steadily increasing in abundance/range in two subpopulations (Scheppers et al., 2013, 2014; Vervaeke, 2012). Wild boar in Belgium have home ranges, expansion velocity and subadult dispersal that are representative of Belgian community size: see the detailed description and references in Chapter VI.

TBE reservoir rodents are ubiquitous and abundant with a suitably limited home range (300-2,500 m^2) for TBEV surveillance. As the reservoir, they develop high-grade viraemia, produce TBEV-specific antibodies (Imhoff et al., 2015; Tonteri et al., 2013) and can be persistently infected. Sero-prevalence studies in rodents show good correlation with local human TBE incidence (Achazi et al., 2011; Imhoff et al., 2015; Knap et al., 2012). These arguments make them good targets for attempts to isolate locally circulating TBEV-strains. **Wild rodents** have therefore frequently been studied in endemic areas (Table I-4). Rodents are promising sentinels particularly in areas of low TBEV circulation, as they are ubiquitous, show persistent TBEV-infection and they offer good proxies for human incidence (Achazi et al., 2011). Ecological studies of rodent reservoirs have also been found useful in the assessment of human health risks from other rodent-borne diseases (Zizi et al., 2002).

In **birds** fewer studies have been done (Table I-4) (Juricova and Hubalek, 1999; Mikryukova et al., 2014). In wildlife surveys, it must not be forgotten that an adequate sample size is needed to arrive at valid conclusions, especially at low expected prevalence (Gerth et al., 1995; Perkins et al., 2003). Common and migratory **songbirds** may carry ticks/TBEV south from endemic areas in Russia and Scandinavia (Kazarina et al., 2015; Lommano et al., 2014; Waldenström et al., 2007). Blackbirds, robins, song thrushes, winter wrens and great tits have proved to be important *I. ricinus* hosts and reservoirs in Borreliosis natural cycles, including in Belgium (Heylen et al., 2013; Heylen et al., 2014; Kipp et al., 2006; Marsot et al., 2012).

Other wild mammals have been investigated on occasion, incuding moose (*Alces alces*), Bison (*Bison bonasus*), European Brown hares (*Lepus europaeus*), Mouflon (*Ovis musimon*), and Chamois (*Rupicapra rupicapra*). Even monkeys, small mammals, reptiles and amphibians have been studied (Table I-4).

Table I-4: Overview of veterinary sentinel studies in wild animals				
Species	***Country***	***Test***	***Prevalence and sample size***	***References***
Roe deer - *Capreolus capreolus*	Belgium	SNT	4.90% (n=98; Flanders)	(Tavernier et al., 2015)
		ELISA - SNT	12% - 0.4% (n=498; Wallonia)	(Linden et al., 2012)
	Czech Republic	HIT	10.9% (n=55)	(Juricova and Hubalek, 1999)
		HIT	21% (n=33)	(Hubalek et al., 1993)
	Austria	IFAT	2.4% (n=945)	(Duscher et al., 2014)
		HIT	15.2% (n=223)	(Radda et al., 1968)
	Germany	SNT	22.9% (n=105)	(Kiffner et al., 2012)
		ELISA - HI - SNT	26% - 24.5 % - 22% (n=192)	(Gerth et al., 1995)
		ELISA - IFAT - SNT	22.86% (n=35)	(Balling et al., 2014)
	Croatia	HIT	24% (n=37)	(Borcic et al., 1990)
	Poland	ELISA	9.1% (n=11)	(Cisak et al., 2012)
	Sweden	ELISA	50.0% (n= 22)	(Gómez-Martínez, 2014)
	Denmark	HIT	8.7% (n=237)	(Skarphedinsson et al., 2005)
	Netherlands	ELISA - HIT - SNT	0.0%- 0.0%- 0.0% (n=13)	(van der Poel et al., 2005)
Fallow deer – *Dama dama*	Czech Republic	HIT	12.0% (n=209)	(Juricova and Hubalek, 1999)
		HIT	0% (n=4)	(Hubalek et al., 1993)
	Poland	ELISA	15.0% (n=14)	(Cisak et al., 2012)
	Sweden	ELISA	25.0% (n= 60)	(Gómez-Martínez, 2014)
Red deer	Austria	HIT	22.6% (n=31)	(Radda et al., 1968)
Cervus elaphus	Czech Republic	HIT	10.7% (n=56)	(Juricova and Hubalek, 1999)
		HIT	9% (n=24)	(Hubalek et al., 1993)
	Sweden	ELISA	41.7% (n= 24)	(Gómez-Martínez, 2014)
	Poland	ELISA	8.3% (n=12)	(Cisak et al., 2012)
	Croatia	Nested RT-PCR	1.1% (n=182)	(Jemersic et al., 2014)
		HIT	39% (n=102)	(Borcic et al., 1990)
Deer	Poland	Nested RT-PCR	2.7% (n=43 roe + red + fallow)	(Cisak et al., 2012)
	Slovakia	HIT - SNT	27.8% (n=18) - 35.3% (n=190)	(Labuda et al., 2002)

Table I-4 (Cont.): Overview of veterinary sentinel studies in wild animals				
Species	*Country*	*Test*	*Prevalence and sample size*	*References*
Wild boar *Sus scrofa*	**Belgium**	**ELISA - SNT**	**4.20% (n=238)**	**Roelandt et al., accepted**
	Czech Republic	HIT	10.0% (n=150)	(Juricova and Hubalek, 1999)
		HIT	6% (n=34)	(Hubalek et al., 1993)
	Poland	ELISA	16.8% (n= 95)	(Cisak et al., 2012)
	Sweden	ELISA	32.0% (n= 122)	(Gómez-Martínez, 2014)
	Croatia	HIT	39% (n=81)	(Borcic et al., 1990)
	South Korea	Nested RT-PCR	0% (n=16)	(Kim et al., 2008)
	Netherlands	ELISA - HIT - SNT	7% - 0% - 0% (n=666)	(van der Poel et al., 2005)
	Slovakia	HIT/SNT	36.8% (n=38)	(Labuda et al., 2002)
	Austria	HIT	1/2	(Radda et al., 1968)
	Germany	ELISA/IFAT/ SNT	10.26% (n=1,851)	(Balling et al., 2014)
Moose *Alces alces*	Sweden	ELISA	41.9% (n= 31)	(Gómez-Martínez, 2014)
Bison *Bison bonasus*	Poland	Nested RT-PCR	0% (n=95)	(Biernat and Karbowiak, 2014)
Brown hare *Lepus europaeus*	Czech Republic	HIT	3.6% (n=193)	(Juricova and Hubalek, 1999)
		HIT	2% (n=48)	(Hubalek et al., 1993)
	Croatia	HIT	0% (n=25)	(Borcic et al., 1990)
	Slovakia	SNT	3.7% (n=596)	(Labuda et al., 2002)
	Austria	HIT	2/13	(Radda et al., 1968)
Mouflon *Ovis musimon* **or Chamois** *Rupicapra rupicapra*	Czech Republic	HIT	7.5% (n=80 mouflon)	(Juricova and Hubalek, 1999)
		HIT	0% (n=2 mouflon)	(Hubalek et al., 1993)
	Slovakia	HIT	3.8% (n=79 mouflon)	(Labuda et al., 2002)
	Austria	HIT	1/3 chamois	(Radda et al., 1968)
Fox *Vulpes vulpes*	Germany	ELISA SNT-WB	2.9 % border and 0.5% pos - 0.13% pos (n=786)	(Wurm et al., 2000)
	Germany	ELISA	1.8-34.2% ~ region (n total = 473)	(Rieger et al., 1999)
	Netherlands	ELISA - HIT - SNT	0.5% - 0% - 0% (n=399)	(van der Poel et al., 2005)
	Austria	HIT	38.5% (n=26)	(Radda et al., 1968)
		HIT	0/4	(Sixl et al., 1973)

Table I-4 (Cont.): Overview of veterinary sentinel studies in wild animals				
Species	***Country***	***Test***	***Prevalence and sample size***	***References***
Small Rodents	Slovakia	Serology	14.6% (3.3-18.1% ~spp.) (n=2,922)	(Kozuch et al., 1990)
	South Korea	Nested RT-PCR	16.6% (n=24)	(Kim et al., 2008)
	Hungary	RT-PCR	4.2% (n=405)	(Pinter et al., 2014)
		SNT	5.19% (n=539)	(Zöldi et al., 2014)
	Croatia	IFAT / real-time RT-PCR	0.0% / 0.0% (n=194)	(Svoboda et al., 2014)
	Germany	Real-time RT-qPCR	10.2% (n=441)	(Achazi et al., 2011)
		Nested RT-PCR	0% (n=300 spleens + 59 brains)	(Kießling, 2005)
	Russia	Nested RT-PCR - ELISA – HIT	61.4% -60.3% (n=3921) 0.65% (*Sorex*) ; 1.65% (*Apodemus*) ; 4.87% (*Clethrionomys*)	(Bakhvalova et al., 2006)
	Italy	PCR/ELISA	0% (n=108 *C. glareolus*) ; 3.3% (n=238 *A. flavicollis*)	(Rizzoli et al., 2004b)
	Finland	IFAT - real-time RT-PCR	4%- 11.9% (n= 202)	(Tonteri et al., 2011)
	Austria	SNT	47.9% (n= 47 *A. flavicollis*); 29.4% (n=34 *C. glareolus*)	(Labuda et al., 1993e)
		HIT	21.43% (n=42 *Apodemus spp.*); 7/13 *C. glareolus*; 1 /2 *M. minutus*; 0/1 *M. musculus*	(Sixl et al., 1973)
	Slovenia	IFAT	5.9% (n=1,401)	(Knap et al., 2012)
	Netherlands	ELISA - HIT - SNT	0.0% (n=90)	(van der Poel et al., 2005)
	Finland	Isolation	7 strains = 1.92% (n=52 *Sorex, Clethrionomys, Apodemus, Microtus*)	(Brummer-Korvenkontio et al., 1973)
	Switzerland	IFAT + avidity	0%-9.9% (n= 63-333 ~region)	Burri et al., 2012 (Burri et al., 2012)
Birds	Czech Republic	HIT	4 strains = 7.4% (n=162 *P. colchicus*)	(Juricova and Hubalek, 1999)
	Russia	RT-PCR - Ag-capture EIA	9.7% - 22.8% (n=779 diverse spp.)	(Mikryukova et al., 2014)
	Finland	Isolation	2.84% (n=161 diverse spp.)	(Brummer-Korvenkontio et al., 1973)
		Hemi-nested RT-PCR	0% (n=100 waterfowl cloacal/tracheal swabs)	(Lindh et al., 2008)

Table I-4 (Cont.): Overview of veterinary sentinel studies in wild animals				
Species	***Country***	***Test***	***Prevalence and sample size***	***References***
Monkeys	Germany	ELISA/SNT	2.6% (n=283 *Macaca Sylvanus*)	(Klaus et al., 2010a)
Other small mammals	Austria	HIT	0/3 wild cats; 0/1 badger; 0/1 weasel	(Radda et al., 1968)
		HIT	Positive: 0/19 bats; 0/2 marmots ; 0/6 hamsters; 6/61 hedgehogs; 3/14 ground squirrels; 2/6 rabbits	(Sixl et al., 1973)
Reptiles and Amphibians	Austria	HIT	Positive: 1/15 lizards; 10/39 snakes	(Sixl et al., 1973)
	Finland	Isolation	0/8 reptiles; 0/12 amphibians	(Brummer-Korvenkontio et al., 1973)

I.5.2.3 Influential factors

It is important to note that some factors are known to influence the seroprevalence observed in animals in veterinary surveillance programs:

Longevity of antibodies has not been extensively assessed in animals, but in most TBEV-exposed dogs a strong IgG immune response is detectable for >2 months in CSF to 9 months in serum (Bjöersdorff, 2002; Leschnik et al., 2002; Rendi-Wagner, 2004), though the exact antibody lifespan is not known (Pfeffer and Dobler, 2011). In sheep and goats, TBEV-specific antibody titers could be detected for >1 year or up to 28 months after respectively natural infection or four immunizations with a commercially available TBEV vaccine (Klaus et al., 2011; Klaus et al., 2014).

Seroprevalence rates are higher in **older animals due** to higher lifetime exposure: dogs, cattle, sheep and horses (Janitza-Futterer, 2003; Klaus et al., 2013; Sîkutova et al., 2009; Vesenjak-Hirjan et al., 1976a, b, c, d), but with a decrease in the oldest horses (Rushton et al., 2013). The **season** may play a role in cattle, with observation of higher seroprevalence during tick season (summer) (Juceviciene et al., 2005; Sîkutova et al., 2009) and large **breeds** seem to be more exposed in dogs (Janitza-Futterer, 2003).

Male horses may have higher tick infestation rates due to frequent transfers to other regions or due to unknown biological reasons (Rushton et al., 2013). A preferential attraction of ticks to male hosts and the 20/80 rule (the observation that 20% of the hosts carry 80% of the vectors or parasites) were also observed in the yellow-necked mouse (*Apodemus flavicollis)* (Burri et al., 2011; Perkins et al., 2003; Randolph and Green, 1999; Randolph et al., 1999) and in fallow deer, though the opposite has been found in moose, roe deer and wild boar (Gómez-Martínez, 2014).

I.5.3 VECTOR SURVEILLANCE

Tick surveillance by PCR testing has been performed in a large number of studies. Ticks have the advantage of offering direct evidence of TBEV presence and some authors have found tick surveillance useful to describe natural foci (Klaus et al., 2010b; Oehme et al., 2002; Süss et al., 2002). Ticks are usually collected in the field by blanket dragging or alternatively collected from human patients or animal hosts (Drelich et al., 2014; Falco and Fish, 1992; Klaus et al., 2010b; Süss et al., 2004). These studies are summarized in Tables I-5 and I-6.

However, tick studies have shown very variable TBEV **prevalence** (Mansfield et al., 2009), with estimates ranging from 0-5% (Dumpis et al., 1999; Gaumann et al., 2010; Oehme et al., 2002; Randolph, 2011), which is often remarkably **lower** than in sentinel species serosurveillance in the same endemic areas (Andreassen et al., 2012; Brinkley et al., 2008; Drelich et al., 2014). Ticks may even not show any positivity for many years, leading to sudden "re-emergence" of natural foci after very long intervals (up to 15 years) of apparent non-activity (Frimmel et al., 2014). The prevalence may be significantly higher in engorged ticks removed from hosts, up to >40% (Bormane et al., 2004; Süss et al., 2006; Süss et al., 2004).

Testing large numbers (1,000s) of collected questing ticks cannot consistently assure virus detection in well-known endemic foci in many studies (Stefanoff et al., 2013). This may partly be due to inclusion in the analysed tick pools of ticks from uninfected areas or immature ticks, and/or due to shortcomings in the laboratory tests (Pettersson et al., 2014). Indeed, only a small percentage of the overall tick population has a high enough viral load to reach the detection limit in PCR (Süss, 2003), which suggests that the proportion of infected ticks in the study area may be much higher than estimated by insensitive or not appropriately validated PCR assays (Drelich et al., 2014).

Since it often lacks sensitivity, PCR screening of ticks cannot be recommended as sole assessment of human TBE risk without long-term sentinel serology (Klaus et al., 2010b; Rizzoli et al., 2014; Stefanoff et al., 2013). In practice, TBE risk areas, cases and outbreaks can only be accurately explored if all available direct and indirect epidemiological evidence is collected and analyzed together: data from humans, ticks, reservoir rodents, and wild/domestic sentinel animals (Gaumann et al., 2010; Holzmann et al., 2009; Klaus et al., 2010a; Klaus et al., 2010b; Klaus et al., 2013; Leutloff et al., 2006; Süss, 2011).

Table I-5: Overview of prevalence studies in questing ticks
MIR: Minimum Infection Rate: number of positive pools/total number of ticks assayed, with the assumption that only one tick in each pool is positive.

Species	*Country*	*Test*	*Prevalence and sample size*	*References*
Questing Ixodid Ticks	Poland	Nested RT-PCR	1.6% (*I. ricinus*) (n=875) 10.8% (*D. reticulatus*) (n=148)	(Wójcik-Fatla et al., 2011)
		Real-time RT-PCR	0.31% (0.19-1.11%) (n= 4,350)	(Drelich et al., 2014)
		Real-time RT-PCR	0.00% (n=7,436)	(Stefanoff et al., 2013)
		RT-qPCR	MIR: 0.21% (n=7,270 *I. ricinus* in pools) ; MIR: 0.33% (n=600 *D. reticulatus* in pools)	(Katargina et al., 2013)
		Nested RT-PCR	MIR: 0.96% (n=2,075 *I. ricinus*)	(Biernat et al., 2014a)
		Nested RT-PCR	2.12% (0.99-12.5% ~region) (n=471 *D. reticulatus*)	(Biernat et al., 2014b)
		Real-time RT-PCR	MIR: 0.11% (n=5,160 *I. ricinus* in 157 pools)	(Cuber et al., 2015)
	South Korea	Nested RT-PCR	0.6% (n=635 ticks) 10.25% (n= 38 pools) (*I. nipponensis* + *H. longicornis*)	(Kim et al., 2008)
		Nested RT-PCR	0.08 (n=13,053 ticks ; 1,292 pools) ; MIR: 0.06% (*H. longicornis*), 0.17% (*H. flava*), 2.38% *(I. nipponensis)*	(Yun et al., 2012)
	Lithuania	Nested RT-PCR	0.1-1.7% (n= 3,234 ticks) ; 1.4% (n=436 pools)	(Han et al., 2005)
		Real-time RT-PCR	1.38% (range 1.03-16%)(n=3,234 ticks - 436 pools)	(Juceviciene et al., 2005)
		RT-qPCR	MIR: 0.30% (n= 1,990 *I. ricinus* in pools)	(Katargina et al., 2013)
	Estonia	RT-qPCR	MIR: 0.46% (n= 2341 *I. ricinus*) ; 4.23% (n= 946 *I. persulcatus*)	(Katargina et al., 2013)
	Sweden	RT-PCR	0.10-0.51%(n=2,074 nymphs) ; 0.55-4.48% (n=906 adults)	(Pettersson et al., 2014)
		Real-time RT-PCR	0.10-0.42% (n=7,630)	(Brinkley et al., 2008)

Table I-5 (Cont.): Overview of prevalence studies in questing ticks
MIR: Minimum Infection Rate: number of positive pools/total number of ticks assayed, with the assumption that only one tick in each pool is positive.

Species	*Country*	*Test*	*Prevalence and sample size*	*References*
Questing Ixodid Ticks	Finland	Nested RT-PCR	0.34% (n=589 ticks)	(Han et al., 2001)
		Isolation	MIR : 0% (n=183) ; 0.29% (n=12,001); 0.32% (n=9,063)	(Brummer-Korvenkontio et al., 1973)
	Finland/ Russia	RT-PCR	1.00% (n=2,411)	(Jääskeläinen et al., 2010)
	Russia	RT-PCR	5.7% (*I. persulcatus* + *I. pavlovskyi*) (n=?)	(Chausov et al., 2010)
	Germany	Nested RT-PCR	0-5.3% ~region/lifestage (n=15,400 *I. ricinus*)	(Süss et al., 2002)
		PCR	0.38% (nymphs) - 6.9% (n=820) ; 1.17% (adults) - 9.3% (n=90)	(Süss et al., 2004)
		Nested PCR	0- 2.3% (n= 9,189)	(Oehme et al., 2002)
		PCR	0.12% (95% CI: 0.05-0.44%) (n=1,657)	(Holbach and Oehme, 2002)
		Nested RT-PCR	0.4±4.8%	(Süss et al., 1999)
		Nested RT-PCR	2.4% (n=250)	(Frimmel et al., 2010)
		Nested RT-PCR	0.0% (n=16,089) -Between 1992-2003	(Frimmel et al., 2014; Klaus et al., 2010b)
		Nested RT-PCR	2.7% (n=150)	(Frimmel et al., 2014)
		Nested RT-PCR	0% (n=212 pools)	(Kießling, 2005)
		Real-time RT-PCR	0.00% (n>16,000)	(Stefanoff et al., 2013)
		Real-time RT-PCR	0.08% of pools ; adult females: 1.28% ; (n= 9,115 ticks - 505 pools)	(Bingsohn et al., 2013)
		Real-time RT-PCR	0.00% (n=3,741)	(Klaus et al., 2010b)
		Real-time RT-PCR / RT-qPCR	0% (n=294 unfed)	(Klaus et al., 2010a)
		Real-time RT-PCR	0.06% (n=1,700)	(Klaus et al., 2012)
	Norway	Real-time RT-PCR	0.53% pools (n=563) pools); 0.11-1.22% ticks (n=5,630)	(Andreassen et al., 2012)
	Netherlands	Real-time RT-PCR	0% (n=906)	(van der Poel et al., 2005)
	Spain	Real-time RT-PCR	0% (n=1,800 nymphs in pools + 630 adults)	(Barandika et al., 2010)
	Luxemburg	RT-PCR	0% (n=1,394)	(Reye et al., 2010)
	Mongolia	RT-PCR	1.32% (n=680 *I. persulcatus*)	(Muto et al., 2015)
		Real-time RT-qPCR	0%/0.68% n=1,557 ticks	(Klaus et al., 2013)

Table I-5 (Cont.): Overview of prevalence studies in questing ticks
MIR: Minimum Infection Rate: number of positive pools/total number of ticks assayed, with the assumption that only one tick in each pool is positive.

Species	*Country*	*Test*	*Prevalence and sample size*	*References*
Questing Ixodid Ticks	Austria	Isolation	0.44% (n=3,404); MIR: 4.4/1000 ticks	(Labuda et al., 1993e)
		RT-PCR	0% (n=306)	(Dobler et al., 2008)
	Hungary	Semi-nested RT-PCR	0.08% (n=2,300 unfed nymphs)	(Pinter et al., 2013)
		RT-PCR	0.00% (n= 1,800)	(Egyed et al., 2012)
		Mouse Inoculation Test + Isolation	3 pools (n=9,616)	(Zöldi et al., 2014)
	Czech Republic	IFAT	0.2-1.3% (nymphs) ; 5.9-11.1% (adults) ; MIR: 0.6% (187 pools; 2,968 ticks)	Danielova et al., 2002
		Isolation	11 isolates (n=2,*157 I. ricinus*); 0.0-4.5% (n=100-640 ♀~region); 0.3-2.2% (n=60-210 ~region)	Asmera and Heinz, 1972
		Real-time RT-PCR	0.32% (nymphs) - 0.81% (adults) (n= 20,057 *I. ricinus*)	Hönig et al., 2015
	Latvia	PCR	*I. ricinus* : 1.7 -26.6% (adults) ; 43% (nymphs) (n=?) *I. persulcatus* : 0 -37.3%. (adults) (n=?)	Bormane et al., 2004
		Nested RT-PCR	2.8% (n=525 *I. ricinus*); 5% (n=281 *I. persulcatus*)	Süss et al., 2002
		RT-qPCR	MIR: 1.02% (n= 3,812 *I. ricinus* in pools); 1.74% (n= 287 *I. persulcatus*)	Katargina et al., 2013
	Estonia	RT-qPCR	MIR: 0.46% (n= 2341 *I. ricinus*) ; 4.23% (n= 946 *I. persulcatus*)	Katargina et al., 2013
	Italy	Real-time RT-PCR	1.2% (0.5-2.5%) (n=1,739)	Carpi et al., 2009
		IFAT/RT-PCR	0.03% (n=13,007)	Hudson et al., 2001
		Real-time RT-PCR	2.1% (n=193)	Capelli et al., 2012
		Nested RT-PCR	0.21% (n=2,361)	D'Agaro et al., 2009
	Switzerland	Nested RT-PCR	14.3% (n=307)	Casati et al., 2006
		Real-time RT-PCR	0.46% (n= 62,343)	Gäumann et al., 2010
		RT-qPCR	1.99% (95% CL : 0.33-6.06) (n=?)	Rieille et al., 2013
		RT-qPCR	0.16-11.11% ~ region (n total = 19,331)	Rieille et al., 2014
		Real-time RT-PCR	MIR: 0.1% (n=6,683)	Burri et al., 2011
		Real-time RT-PCR	0.1% (n= 6,120)	(Lommano et al., 2012)

Table I-6: Overview of prevalence studies I nticks collected off hosts.
MIR: Minimum Infection Rate: number of positive pools/total number of ticks assayed, with the assumption that only one tick in each pool is positive.

Species	*Country*	*Test*	*Prevalence*	*References*
Foxes	Croatia	Nested RT-PCR	1.6% (n=371)	(Jemersic et al., 2014)
Rodents	Hungary	Semi-nested RT-PCR	0.78% (n= 431)	(Pinter et al., 2013)
	Switzerland	Real time RT-PCR	MIR: 0.1% (n=3,303); 8.6%-50% (n=77 off 2 rodents)	(Burri et al., 2011)
Cervids	Germany	Real time RT-PCR	0.00% (n=187)	(Klaus et al., 2010b)
Roe deer	Italy	RT-PCR/IFAT	0.23% (n=878)	(Hudson et al., 2001)
Dogs / Cats	**Belgium**	**TBEV not tested**	**(n=2,373)**	(Claerebout et al., 2013)
Birds	Sweden	Nested RT-PCR	0.52% (n=1,155)	(Waldenström et al., 2007)
	Russia	RT-PCR	14.1% (*I. persulcatus*) ; 5.2% (*I. pavlovskyi*) ; 4.2% (*I. plumbeus*)	(Mikryukova et al., 2014)
	Switzerland	RT-qPCR	0.27% (n=1,123)	(Lommano et al., 2014)
	Estonia	Real time RT-PCR	0.4% (n=249)	(Geller et al., 2013)
	Latvia	RT-qPCR	14% (n=170)	(Kazarina et al., 2015)
	Moldova	PCR	0.7% (n= 135 *I. ricinus*)	(Movila et al., 2013)
Humans	Germany	Real time RT-PCR	1.3% (n= 239)	(Klaus et al., 2010b)
		Nested RT-PCR	8.8% (n=561)	(Süss et al., 2006)
		PCR	8.4% (n=215)	(Süss et al., 2004)
	Sweden/Finland	Multiplex real-time RT-qPCR	0.23% (n=2,167)	(Lindblom et al., 2014)

I.5.4 Risk Maps

TBE risk maps based on reported human TBE cases are published regularly (Figure I-11). These maps give a good but incomplete picture of the European situation, as they usually do not take into account the tick and animal prevalence of TBEV (Rendi-Wagner, 2004; Süss, 2003).

Alternatively, TBE risk maps may also be based on satellite-derived GIS and RS data on environmental and climatic characteristics and based on virus and host survival requirements. Such maps can predict TBE risk areas with 85% accuracy (Figure I-12) (Labuda and Randolph, 1999; Randolph, 2000, 2001; Randolph and Rogers, 2000; Rinaldi et al., 2006).

On the basis of climate change predictive models, it could be speculated that endemic regions may further disperse geographically in any one, two or even three directions − eastwards, northwards and/or even westwards (Randolph, 2001; Randolph and Rogers, 2000), as observed in Sweden, Germany and France (Kirtz, 1999; Lindgren and Gustafson, 2001; van der Poel et al., 2005).

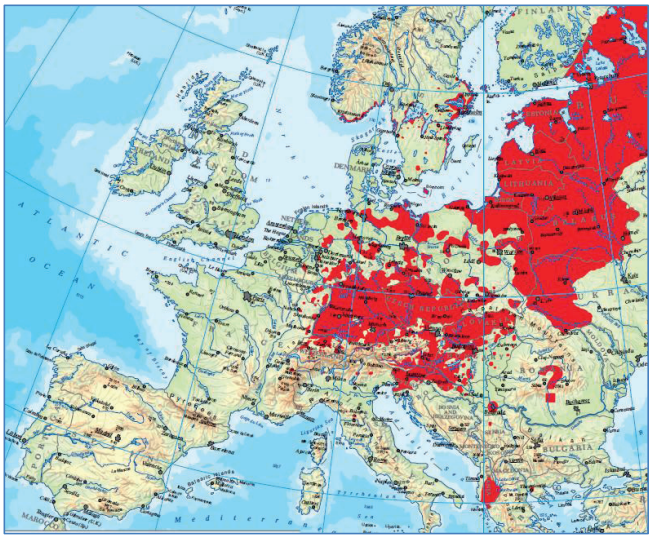

Figure I-11: TBE in Europe: Established endemic areas in 2013.
Red zones: TBE risk area; Question mark: potential TBE risk area; Adapted from ISW-TBE, 2013.

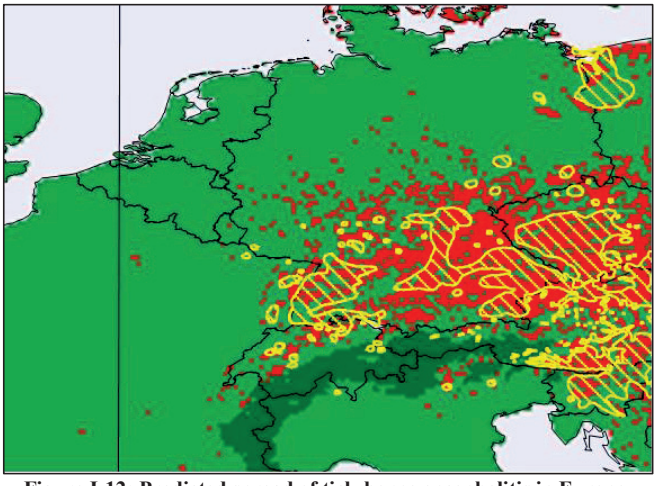

Figure I-12: Predicted spread of tick-borne encephalitis in Europe.
Red zones: satellite-derived predicted distribution (data 2000) compared with Yellow zones: established foci (mapped 1997); Randolph and Rogers, 2000; Randolph, 2000; permission The Royal Society and S. Randolph.

I.6 STATE OF THE ART IN BELGIUM ANNO 2009-2016

I.6.1 TICK (BITE) SURVEILLANCE

In 2015, a human tick bite notification system for the Belgian general public was launched (https://tekennet.wiv-isp.be/; Figure I-13)(Lernout, 2016; WIV-ISP, 2015).

The presence of ***Ixodes ricinus*** **ticks** has now been confirmed throughout the whole Belgian territory (Claerebout et al., 2013; ECDC et al., 2015; Obsomer et al., 2013). In fact, this tick species seems to have been omni-present long before 2011, as recently demonstrated by the mapping of 14 tick collections from different institutes, musea and surveys, and field observations by Natuurpunt and Natagora (www.natuurpunt.be; www.natagora.be)(Obsomer et al., 2013).

The temporal distribution in Figure I-14 seems to follow that in other countries (ECDC et al., 2015; Obsomer et al., 2013). Some other Ixodid ticks present in Belgium are *Dermacentor reticulatus* (in a patchy distribution), *Rhipicephalus sanguineus* (very localized and imported) and *Ixodes hexagonus* (widely distributed) (Claerebout et al., 2013; Obsomer et al., 2013).

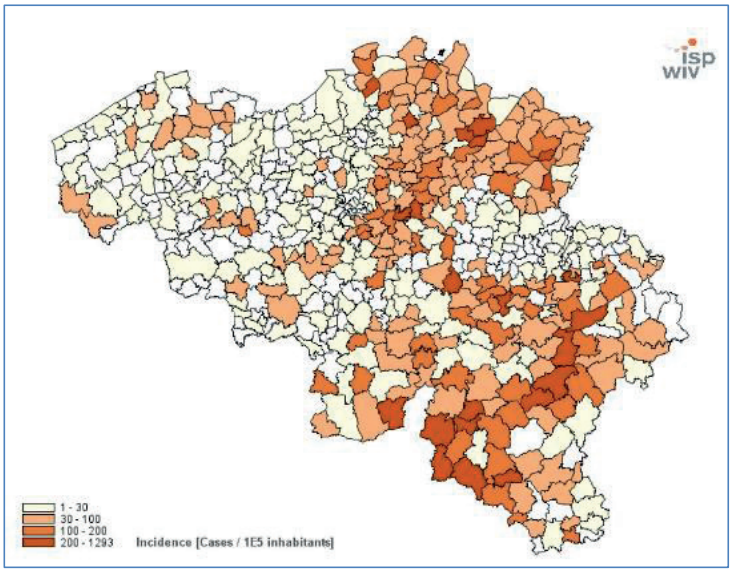

Figure I-13: Incidence of reported tick bites by municipality, July-Dec 2015.
https://tekennet.wiv-isp.be ; (Lernout, 2016)

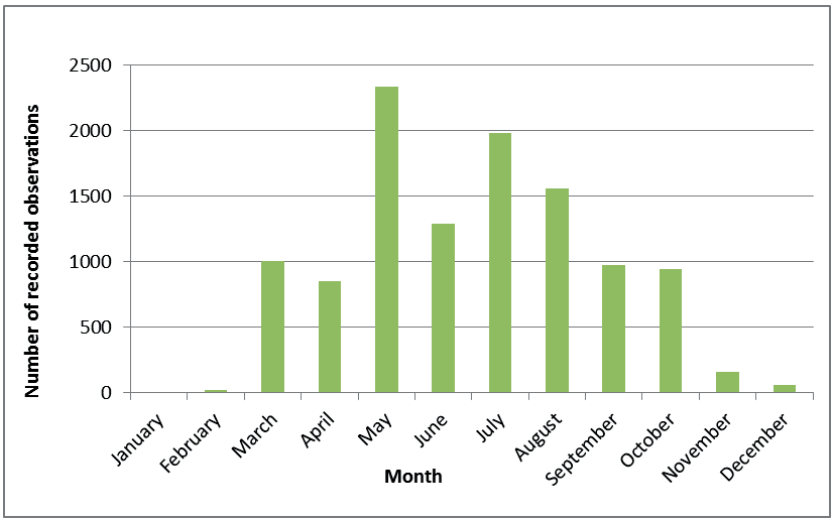

Figure I-14: Temporal distribution of *I. ricinus* ticks in Belgium.
Data compiled from several tick collections and studies and adapted from table 4 in Obsomer et al. (2013).

To date, there is no evidence of ticks carrying TBEV in Belgium, however, no published studies have actually tested for this pathogen. Several other **pathogens** have been found in Belgian ticks, such as *Borrelia burgdorferi s.s., B. valaisana, B. garinii, B. afzelii, B. spielmani* and *B. lusitaniae* (Claerebout et al., 2013; Estrada-Pena et al., 2011).

Anaplasma phagocythophilum and *Rickettsia helvetica/massilae* seem to be present throughout the country (Claerebout et al., 2013; Lempereur et al., 2012; Obsomer et al., 2013), while occasionally *Babesia* spp. have been detected, e.g. *B. divergens* (Everaert et al., 2007; Famerée et al., 1977; Lempereur et al., 2012; Losson, 1989; Saegerman et al., 2007), *B. canis* and *B. caballi* (Jongejan et al., 2015; Losson et al., 1999), *B. microti* and *B. EU1* (Lempereur et al., 2011). Tick and pathogen diversity hotspots may be present in several communities (Obsomer et al., 2013).

I.6.2 MEDICAL TBE SURVEILLANCE

During 2000-2010, no autochthonous TBE cases were reported and TBE was not known to be endemic in Belgium, despite having climatic and environmental conditions that are conducive to the circulation of the TBE virus (ECDC, 2012). In this decade, there was only one published paper which tried to identify TBE in four human patients with a viral CNS infection of unknown etiology from Belgium. None of the four Belgian patients included in this study were confirmed as a TBE case (Haglund et al., 2003). During the same period, the **Queen Astrid Military Hospital** performed a continuous serological screening with the Virotech® IgG/IgM ELISA (Sekisui Diagnostics - Genzyme Virotech) in 359 suspected tick bite and neurology patients between 07/2000 and 10/2011. Of these, 55 tested IgG positive (15.32%) and 19 tested IgM positive (5.29%). However, none of these results could be confirmed in SNT (P. Heyman, personal communication, 2015).

Since 2010, the TBE National Reference Centre **(WIV-ISP)** has used the European case definition (EC, 2012; ECDC, 2012) and has offered serological and PCR screening to the medical sector as part of a **diagnostic** service of the referral laboratory and a limited passive surveillance system with voluntary reporting of CNS cases (ECDC, 2012). During 2010-2016, the Belgian TBE-NRC (WIV-ISP) has been using Progen Immunozym FSME/TBEV IgM and IgG kits to screen human patients (Progen, 2014; Progen, 2012) and the TBEV-SNT as a confirmation test (Vene et al., 1998). SNT-results from ≥1/10 onwards are considered sufficiently protective against clinical TBE, but titers are usually much higher after full vaccination (Holzmann et al., 1996; Kollaritsch et al., 2011b; WHO, 2011). Additionally, comparative IFA Biochips (Mosaic 3, Euroimmun®, Germany)(Litzba et al., 2014) and qRT-PCR (Schwaiger and Cassinotti, 2003) have been available at the TBE-NRC and current best practices in TBE-diagnostics are followed.

As such, TBE tests have been performed on patients suspected of neuroborelliosis and on cases that were sent by general practitioners or hospitals based on direct TBE clinical suspicion (Dr. Van Gucht S., and Dr. Brochier B., pers. comm., 2015). In 60 samples from 2009, 10 reacted in IgG ELISA (borderline or positive), one was positive in IgM-ELISA and seven reacted positive in RFFIT-SNT. In 2014, 53 suspected patients were tested and a total of 18 samples were IgG-ELISA-positive or -borderline, while three were SNT-positive, but none were IgM-ELISA-positive.

No samples were positive for all three tests together, and since convalescent (paired) samples were not available and the TBE/flavirirus vaccination status of the patients is unknown, the interpretation of these results remains inconclusive. So far, six imported cases of human TBE imported from Scandinavia, Austria, Kyrgyzstan and Slovenia have been confirmed by the Belgian NRC (Dr. Suin V. and Dr. Van Gucht S., pers. comm, 2015).

According to ECDC (2014), Belgium has not yet adopted a comprehensive surveillance system and there is no nationwide active surveillance component in occupational risk groups (ECDC, 2014). The existing passive surveillance is not mandatory and is based on one reference laboratory reporting very fragmentary data with little clinical or vaccination history or follow-up (no paired sera), presented by clinicians on a voluntary basis only.

It is known that TBE notification is not mandatory and is considered unimportant in Belgium (Callens, 2016; Donoso Mantke et al., 2008a; Donoso Mantke et al., 2008b; Randolph, 2001; Süss, 2008b). TBE is a neglected zoonosis as a large number of Belgian neurologists and general physicians are largely unaware of this emerging tick-borne disease as a differential diagnosis to test for human encephalitis/meningitis or tick-bite cases (Dr. P. Roelandt, MD, Dr. M. Goethals, MD Neurologist, Dr. Van Gucht S., DVM, Virologist).

Testing for TBEV in human encephalitis cases is in general not even recommended in diagnostic protocols, unless there are clear indications in the exposure history (Callens, 2016; Solomon et al., 2007). Despite this, the situation may be slowly improving anno 2016, with more clinicians and pharmacists seeking information at the current NRC (Institute of Tropical Medicine, Antwerp) about preventive TBE vaccination (Dr. Soentjens P., Dr. Maniewski-Kelner U., Van den Daele A., Dr. Van Esbroeck M., ITM, pers. comm., 2016). Belgian citizens travel to known endemic areas and may be infected there or may return with infected ticks (Heyman, 2009; Luyasu, 2009), but most likely very few of those tourists are vaccinated: see Table I-7 (Dr. Soentjens P., Dr. Maniewski-Kelner U., Van den Daele A., ITM, pers. comm., 2016).

Vaccination	1999	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015
Adult	88	53	55	58	39	32	69	29	51	38	60
Junior	0	2	1	3	0	3	5	0	1	0	2
Grand Total	**88**	**55**	**56**	**61**	**39**	**35**	**74**	**29**	**52**	**38**	**62**

Table I-6: Number of TBE vaccins (FSME® Baxter) administered by the Belgian Institute of Tropical Medicine.

(ITM, pers. comm., 2016)

I.6.3 WHY BELGIUM NEEDS VETERINARY TBE SURVEILLANCE

Until now, no veterinary or tick screening had been performed, since TBEV is rather a public health problem than a disease of large veterinary importance and is not "believed" to be present. Additionally, not a single human TBE case has been reported in a person from Belgian nationality, despite the fact that:

(1) Belgium has climatic and environmental conditions that are conducive to the circulation of the TBE-virus and similar to those of other European countries where TBEV is endemic (AGIV, 2009; ECDC, 2012; FAO, 2001; KMI, 2009; RW, 2009);

(2) Suitable vectors and hosts are present and populations are abundant or increasing (Claerebout et al., 2013; Misonne et al., 1998; Obsomer et al., 2013; Verkem et al., 2003);

(3) Throughout the past 15 years small numbers of suspected human clinical neurological cases have regularly occurred, that could not yet be completely confirmed through lack of complete epidemiological investigations, while imported cases have occurred and were confirmed (ECDC, 2014);

(4) Animal tick prevention, owner compliance and education is often insufficient (Dryden, 2009; Leschnik et al., 2013). Furthermore, dogs and horses often travel with their owners (Heyman, 2009; Luyasu, 2009), and wildlife (mammals and birds) can easily cross national borders. TBEV-infected ticks can therefore easily be transported into Belgium.

(5) People travel to endemic areas without vaccine protection.

Extensive research by many authors in Europe and Asia on temporo-spatial heterogeneity in TBEV-foci and TBE incidence trends have shown that not detecting human cases at a single time point does not substantiate TBEV absence, as there are many underlying (a)biotic factors that determine the visible human TBE incidence pattern at the tip of the iceberg. Human cases are clearly not sufficient data to completely describe the geographical presence of a TBEV focus or to predict future dynamics (Balmer et al., 2007b; Randolph and Sumilo, 2007). Nonetheless, this knowledge of the TBEV distribution is essential to ensure an appropriate response to the related risks and to decide the appropriateness of vaccination (Holbach and Oehme, 2002).

Consequently, both veterinary and ecological TBE studies have clearly proven their value in known TBE endemic areas and specifically in areas or countries with (very) few human cases and few suspected areas, where one doubts if TBE(V) is even present. The zoonotic iceberg phenomenon has been illustrated in the field on several occasions, e.g. in Scandinavia, Germany and in Japan.

All these studies clearly support the usefulness of preventive veterinary TBE surveillance as an early warning of suspected endemic areas before the tip of the iceberg suddenly surfaces in the form of human cases (Barandika et al., 2010; Broker, 2002; Chiba et al., 1999; Csángó et al., 2004; Fomsgaard et al., 2009; Fomsgaard et al., 2013; Frimmel et al., 2010; Frimmel et al., 2014; Gaumann et al., 2010; Hayasaka et al., 1999; Kristiansen, 2002; Laursen and Knudsen, 2003; Randolph, 2001; Randolph and Sumilo, 2007; Reye et al., 2010; Skarpaas et al., 2004; Skarpaas et al., 2002; Skarphedinsson et al., 2005; Süss et al., 2004; Takashima, 1998; Takashima et al., 2001; Takashima et al., 1997; Takashima et al., 1992; Takeda et al., 1998; Takeda et al., 1999; Takezawa et al., 1995; van der Poel et al., 2005; WHO, 2004).

I.7 CONCLUSIONS OF THE GENERAL INTRODUCTION

"Even though TBE was described as early as 1931 (Schneider, 1931), this dangerous form of encephalitis has been underestimated for a long time" (Kunz, 2008). The absence of any human cases in Belgium (even travel related) is perhaps questionable since Belgian citizens and animals regularly travel to known endemic areas. Furthermore, Belgium has climatic and environmental conditions similar to those of other European countries where TBEV is endemic and suitable vectors and hosts are present and abundant.

However, presently clinicians do not routinely test for TBE(V) and notification of clinical cases is not mandatory. Until now, TBE surveillance in Belgium has been very minimal and fragmented, therefore cases could currently remain undiagnosed and/or the establishment of endemic foci with low prevalence could easily be missed. Just as increased awareness and testing have probably contributed to the rise in Belgian human cases of Lyme disease, so too may increased veterinary surveillance demonstrate the arrival or circulation of TBE(V) in Belgium.

Many countries have led the way and showed the great potential of veterinary surveillance to increase the overall detection sensitivity of national TBEV surveillance schemes and to greatly improve the description of (potential) endemic risk areas, especially in regions where the human cases are emerging, extremely rare and/or tick prevalence is undetectably low.

Until now (2009), veterinary TBE surveillance in Belgium has been non-existent, hence human or veterinary cases may currently remain undiagnosed and the introduction and establishment of endemic foci with low prevalence could easily be missed. Just as increased awareness and testing have probably contributed to the rise in Belgian cases of Lyme disease (Ducoffre, 2008a), so too may increased veterinary and human surveillance demonstrate the arrival or circulation of TBEV in Belgium.

CHAPTER II AIMS OF THE PHD THESIS

Questing for Tick-borne Encephalitis virus in Belgium, using Veterinary Sentinel Surveys and Risk Factor Mapping

In Belgium, there are no confirmed autochthonous TBE cases in humans, despite the perception in the medical community that occasionally suspected cases have occurred in the past and present. However, the etiology often remains unconfirmed and TBEV negative (Dr. Heyman P., pers. comm. 2010 and Dr. Van Gucht S., pers. comm., 2015);(Callens, 2016; Haglund et al., 2003). The ECDC and TBE(V) researchers have stated that in national TBEV-surveillance schemes there is a justifiable and acknowledged need for a veterinary surveillance component to improve and complement the output from surveillance in humans (ECDC, 2012; Süss, 2011).

The goals of this PhD were therefore:

1. To establish veterinary serological evidence pro or contra TBEV presence in Belgium, based on studies in key species relevant for TBEV epidemiology and surveillance → *Chapter III (dogs), Chapter IV (cattle), Chapter V (wild boar) and Chapter VII (general discussion).*

2. To map the geographical distribution of the samples and seropositive cases versus a number of known TBE risk factors → *Chapter VI (mapping and modelling).*

3. To evaluate an ELISA first-line test for veterinary screening based on the gold standard seroneutralisation test → *Chapter III (dogs), Chapter IV (cattle), Chapter V (wild boar), Chapter VII (general discussion).*

4. To evaluate the selected sentinel species (domestic and wild) regarding their suitability for ongoing veterinary TBEV surveillance based on the survey experiences and literature/practical criteria → *Chapter I (introduction), VII (general discussion).*

5. To identify remaining knowledge gaps and suggest priority actions for the future → *Chapter VII (general discussion).*

CHAPTER III SEROLOGICAL SENTINEL SURVEY IN BELGIAN DOGS

Adapted from: Sophie Roelandt, Paul Heyman, Marina De Filette, Sirkka Vene, Yves Van der Stede, Ann Brigitte Caij, Paul Tavernier, Alexandre Dobly, Hendrik De Bosschere, Philip Vyt, Carole Meersschaert, and Stefan Roels

TICK-BORNE ENCEPHALITIS VIRUS (TBEV) SEROPOSITIVE DOG DETECTED IN BELGIUM: SCREENING OF THE CANINE POPULATION AS SENTINELS FOR PUBLIC HEALTH.

Vector-borne and Zoonotic Diseases 2011; 11(10): 1371-1376.

Mary Ann Liebert, Inc. Publishers.

http://www.liebertpub.com/overview/vector-borne-and-zoonotic-diseases/67/

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III.1 ABSTRACT

Tick-borne encephalitis virus (TBEV) is an important emerging tick-borne viral infection of humans and dogs in Europe. Currently, TBEV surveillance is virtually non-existent in Belgium, which is considered non-endemic. A commercial enzyme-linked immunosorbent assay (ELISA) was adapted for the detection of TBEV-specific IgG-antibodies in canine sera. Serum samples of Belgian dogs were obtained from three diagnostic laboratories from Northern (n=688) and Southern Belgium (n=192). ELISA- positive, and borderline and near-borderline samples were subjected to a TBEV rapid fluorescent focus inhibition confirmation test (TBEV-SNT). One dog was confirmed TBEV seropositive. Several ELISA-positive and borderline sera underwent seroneutralization and hemagglutinin inhibition tests to rule out West Nile and Louping Ill viruses, but tested negative. The clinical history of the seropositive dog could not explain beyond doubt where and when TBEV infection was acquired. Further surveillance is necessary to determine whether this dog remains a single travel-related case or whether it represents an early warning of a possible future emergence of TBEV.

III.2 INTRODUCTION

Western subtype tick-borne encephalitis virus (TBEV), transmitted by *Ixodes ricinus* ticks and occasionally by unpasteurized milk from ruminants, is currently the most important arthropod-borne viral infection in humans in Europe (Herpe et al., 2007; Ramelow et al., 1993). In one to two thirds of patients, tick-borne encephalitis (TBE) is a biphasic disease with fever and neurological signs, ranging from meningitis to severe encephalitis with or without myelitis (Haglund and Günther, 2003; Kaiser, 2008b). All patients need hospitalization and approximately 35-58% of patients develop permanent sequelae called “post-encephalitic syndrome” (Haglund et al., 1996; Kaiser, 2008a). Despite low mortality rates of 0-3.9% (Donoso Mantke et al., 2008a; Süss, 2008a), Western subtype TBEV results in very high risk to society and health care (Baumhackl, 2009; Haglund, 2002). TBE is an increasing public health risks in several European countries (Süss et al., 1997) and 3,000 people are hospitalized every year (ECDC, 2012; Haglund, 2002). Recently, incidence of TBEV in humans has been fluctuating and increasing in several endemic countries (Süss, 2008a) and it has emerged in Northern and Western Europe (Donoso Mantke et al., 2008a).

TBE is also emerging among Europe's canine population (Beugnet and Marie, 2009; Leschnik et al., 2002). Small numbers of cases were described in endemic foci of Austria, Switzerland, Germany and Sweden (Bjöersdorff, 2002; Csángó et al., 2004; Gresikova et al., 1972; Kirtz et al., 2001; Reiner and Fischer, 1998; Tipold et al., 1993; Weissenbock et al., 2010). The distribution of canine TBE is steadily expanding over Western Europe in parallel with human TBE. Consequently, a higher number of canine TBE cases are likely to be diagnosed as awareness increases in the veterinary community (Beugnet and Marie, 2009; Leschnik et al., 2002). Although in 50% of dogs seroconversion occurs without any clinical signs (Klimeš et al., 2001; Leschnik et al., 2002), TBEV can cause pyrexia, lethargy, loss of appetite and multifocal neurological signs (Bjöersdorff, 2002). Most dogs develop a strong IgG immune response (Bjöersdorff, 2002; Rendi-Wagner, 2004) which is detectable for more than 2 months in cerebrospinal fluid to 9 months in serum (Leschnik et al., 2002). In analogy to human medicine (Holzmann, 2003; Rendi-Wagner, 2004), the best diagnostic tests for dogs are indirect IgG enzyme-linked immunosorbent assay (ELISA), hemagglutination inhibition (HI), seroneutralization assay (SNT) or immunoblotting tests. SNT tests are considered to be highly specific confirmation / reference tests (Klimeš et al., 2001; Leschnik et al., 2002; Reiner and Fischer, 1998; Vene et al., 1998).

Due to the close relationship between humans and dogs, the latter are a good sentinel species for the spread and risk of TBE. Increased pet travel (Beugnet and Marie, 2009; Leschnik et al., 2002; Otranto and Wall, 2008) combined with more frequent contact between dogs and *I. ricinus* often results in higher seroprevalence rates in dogs as compared to humans (Bjöersdorff, 2002; Klimeš et al., 2001). Dogs may also carry infected ticks from endemic to non-endemic areas and into close vicinity of humans (Grešíková et al., 1972; Leschnik et al., 2002). Sentinel studies in Scandinavian dogs revealed geographical areas where TBE was more common than expected, discovered new TBEV foci and highlighted the need for better TBE(V) surveillance (Bjöersdorff, 2002; Csángó et al., 2004; Skarpaas et al., 2004).

In Belgium, TBE is still considered an exotic disease (Donoso Mantke et al., 2008a; Süss, 2008a) and medical and veterinary TBEV surveillance is currently non-existent (Roelandt et al., 2010). Therefore, we conducted a first serological screening of dogs in Belgium for TBEV.

III.3 METHODS

III.3.1 Sampling and Study Design

Serum samples of Belgian dogs (n=880) were obtained from two diagnostic laboratories from Northern (n=688) and one from Southern (n=192) Belgium (Figure III-1). Afterwards, this sample size was sufficient to adapt the ELISA kit to canine sera and for the purpose of detecting a seroprevalence of 0.35% with a confidence level of 95% (n=855), assuming 100% sensitivity and specificity in dogs. All samples were taken by local veterinary surgeons between 15/03/2009 and 22/06/2009 and submitted to the laboratories for a variety of diagnostic tests. Samples were centrifuged and the sera were stored at 4°C at the laboratories until collected, after which they were frozen and stored at -20°C.

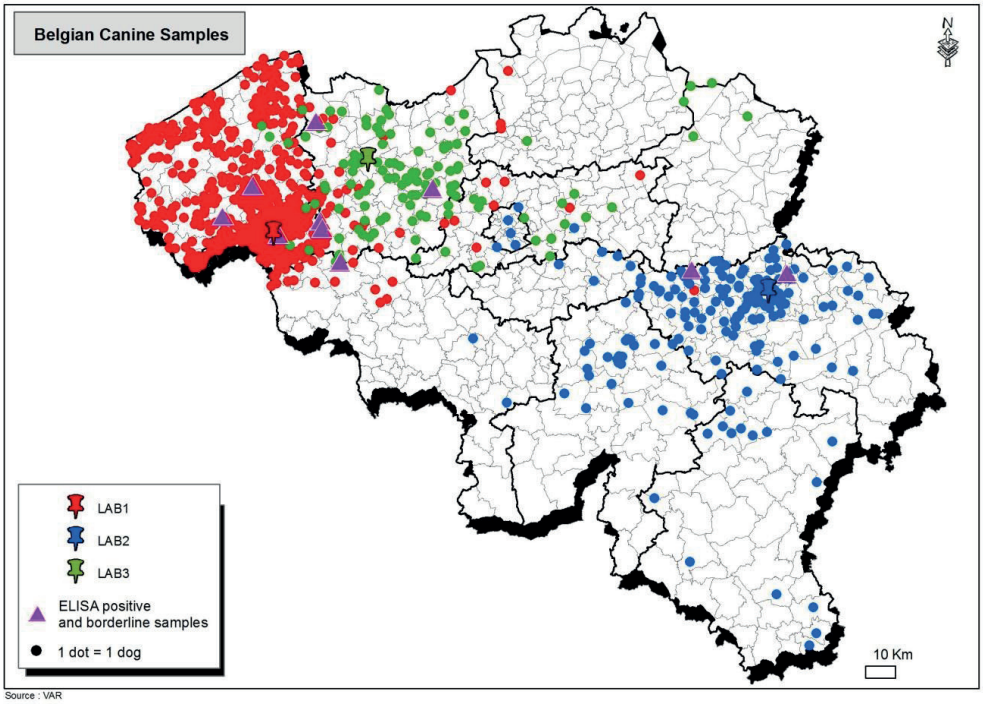

Figure III-1: Belgian canine samples (n=880) used in this study.
Three coloured pins represent the 3 participating laboratories. One dot represents one dog. Purple triangles represent ELISA positive and borderline samples.

III.3.2 DIAGNOSTIC SEROLOGY ASSAYS

III.3.2.1 TBE virus ELISA

A commercially available ELISA test kit (Immunozym FSME/TBE IgG All Species-ELISA®, Progen Biotechnik GmbH, Heidelberg, Germany) was used to detect TBEV- specific IgG antibodies in the canine sera. This non-competitive indirect assay uses horseradish peroxidase – Protein G conjugate to detect IgG against whole TBE-virus. The kit can theoretically be used for TBEV testing in all species, including humans. In humans, this ELISA has a diagnostic sensitivity of 97% and analytical specificity of 99% for IgG (Progen, 2006) and it was previously used as a TBEV screening test in foxes (Wurm et al., 2000).

The kit was adapted using a known positive canine serum from a clinical case (kindly provided by the In Vitro Labor für Veterinärmedizinische Diagnostik und Hygiene GmbH, Vienna, Austria) and a mixture of five TBEV-negative sera (SPF laboratory beagles, Janssen Pharmaceutica N.V., Beerse, Belgium) alongside human calibrator and control samples of the kit. The manufacturer's instructions were followed, sera were diluted 1:50 and optical densities were read at 450 / 620 nm (reference 620 - 690 nm). For each plate, a standard curve was generated using the five human calibrator samples. Sample concentrations were read from these curves in Vienna Units per ml (VIEU/ml). Sera with <53 VIEU/ml were negative, sera with >126 VIEU/ml were positive and those between 53 VIEU/ml and 126 VIEU/ml were classified borderline. Since the true diagnostic sensitivity and specificity of this kit are unknown for dogs, we lowered the cut-off by 15% compared to the kit cut-off (63 → 53 VIEU/ml).

III.3.2.2 Confirmation testing

A serum panel consisting of all ELISA-positive, all borderline and a number of negative sera, was sent for confirmation testing and these sera were used as long as a sufficient volume was available. At the Swedish Institute for Infectious Disease Control (Solna, Sweden), the rapid fluorescent focus inhibition test (TBEV-SNT) was used, essentially as described by Vene (Vene et al., 1998): a mouse monoclonal TBEV-antibody, kindly provided by M. Niedrig, followed by FITC-labelled goat-anti-mouse conjugate (Jackson ImmunoResearch,West Grove, PA) were used to visualize foci. Human control samples were used and antibody titres were calculated as the reciprocal of the serum dilution that reduced the virus to one FFD_{50} (50% focus forming dose). The lowest detectable canine TBE titer is 1/5 (Dr. Vene S., pers. comm. 2009).

The remaining sera were then subjected to a SNT for West Nile virus at the Veterinary and Agrochemical Research Centre (CODA-CERVA, Brussels, Belgium). Briefly, serum dilutions were incubated in a 96-well plate with 100 TCID50 of WNV (IS 98 strain) for 1 hour at 37°C. Chicken control sera (negative/positive) were tested at the same time. After one hour, Vero cells were added to each well and the plate was incubated for 3 days at 37°C with 5% CO2. After 3 days, the cytopathic effect of virus grown was read by microscopy.

A HI test for Louping Ill virus was performed at Moredun Scientific Research Institute (Penicuik, Midlothian, Scotland, UK) as described by Clarke and Casals (1958), but modified to a microtitre plate version, using ovine control samples and a cut off titre of 1:20. Samples were prepared by heat inactivation for 1 hour at 65°C and nonspecific inhibitors and goose erythrocyte agglutinins were removed by kaolin and goose erythrocyte absorption. The LIV-HIT was used because the LIV-SNT was not available at the time. A retrospective descriptive epidemiological investigation was performed for confirmed TBEV ELISA- and SNT-seropositive dogs and consisted of telephonic contact with the owners and the veterinarians to obtain a clinical history.

III.4 RESULTS

III.4.1 DIAGNOSTIC SEROLOGY

Based on the VIEU/ml cut-offs we specified above, the control samples reacted conform expectations clearly positive or negative, two dog sera were found to be ELISA-positive (0.22%) and eight were borderline (0.91%). These 10 samples came from all three of the included diagnostic laboratories and from Northern and Southern. Five ELISA-negative samples were included in the panel of samples for confirmation testing (n=15) that was first sent for SNT testing. Among these, two borderline serum samples belonging to the same dog were found positive by SNT at a low titer (1/5). Hereafter, these two sera are considered as one SNT-positive sample or dog.

Nine borderline/positive ELISA samples (incl. SNT-positive sample) and two of the negative samples were screened for WNV antibodies by SNT: all sera (n=11) were WNV antibody-negative. Five samples (3 borderlines, 1 SNT-positive, 2 negatives) for which sufficient serum remained were additionally tested by LIV-HIT at Moredun Scientific Research Institute. Four samples were LIV-HIT negative, including the TBEV-positive dog. One borderline sample tested LIV-positive (titer 1:160).

III.4.2 Case History

The TBEV (SNT) seropositive dog was an eight-year-old neutered female West Highland White Terrier cross, born in 2001 and living in West Flanders (Northern Belgium, near the red laboratory in Figure III-1). The owners obtained it as a six-week-old puppy and it had been regularly vaccinated for Canine Distemper, Canine Hepatitis, Parvovirus, Parainfluenza, Leptospirosis (DHPPiL or PiL) and Rabies. It was treated against parasites with preventive pyrantel/praziquantel (twice a year) and fipronil (once a year in June). It was never offered unpasteurized dairy products.

Though the dog was diagnosed with hypo-adrenocorticism in 2005, this condition was well controlled by standard treatment with prednisolone and fludrocortisone. The blood samples tested in this study were taken to monitor the animal's status with regard to this condition. According to the owners, this dog never showed any neurological signs or appeared ill, and apart from the Addison's disease episode there was no other disease history. There were no other pets in the household.

This dog had traveled abroad to the German Mosel region in 2002 and to the French Alsace region in 2003. On both occasions, it spent a few weeks in the area, though no forested areas were visited and no ticks were observed at the time. The dog also visited the Ardennes region in Southern Belgium on four occasions during weekend trips in autumn (November). After the end of 2004, the dog had not left the North of Belgium. At home, this dog has access to the back garden and to the local forested countryside in West Flanders.

In the summer of 2008, before the positive blood sample was taken, the owners removed a small red tick from the dog, at home in the garden. Apart from a small crust at the attachment site, the animal remained asymptomatic following the tick bite. Another tick was observed during the summer of 2009, after the diagnostic blood samples included in our study were taken. None of the family members in this household ever showed clinical signs compatible with tick-borne encephalitis.

III.5 DISCUSSION

Our sample of the Belgian canine population has to be considered as a convenience sample. The sera came from all provinces in Northern and Southern Belgium, though they were mainly obtained from four of the ten provinces (Figure III-1). Furthermore, the serum samples were taken either to diagnose illness, to monitor treatment or as pre-anesthetic screening. Therefore, they may not necessarily be representative of the entire canine population in Belgium. Nonetheless, the available sample was of sufficient size to adapt the ELISA kit and to detect a design seroprevalence of 0.35% with a confidence level of 95% (n= 855 needed).

In the case of TBEV testing, all samples found positive or borderline by ELISA need to be confirmed with a reference test such as SNT, since non-specific reactions as well as cross-reactions of TBEV antibodies with other flavivirus antibodies have been described. The same applies for LIV and our results confirm this, as several false positive samples were observed for TBEV and likely also one for LIV, despite adequate sample preparation. It was also clear that the canine cut- off of this ELISA kit needs further evaluation, in order to improve sensitivity and to avoid false negatives.

In humans, cross reactions often occur with Yellow Fever virus, Dengue virus and West Nile virus antibodies (Holzmann, 2003). A cross-reaction between anti-TBEV and anti-WNV antibodies was observed in a dog in the Czech Republic (Klimeš et al., 2001). Therefore, we included WNV as a differential diagnosis for ELISA-positive/borderline but SNT-negative samples.

TBEV is phylogenetically very closely related to LIV and both viruses share I. ricinus as vector. LIV can cause sheep or goat encephalitis and has so far been reported in Scotland, Wales, England, Ireland, Norway, Denmark, Spain, Greece, Turkey and Bulgaria (Dobler, 2010; Grard et al., 2007; Gritsun et al., 2003a). Although few cases have been reported, LIV can occasionally cause encephalitis much like TBE in dogs and humans (Dobler, 2010). The dog that tested positive for LIV-HIT and negative for TBEV-SNT and WNV-SNT, was lost to follow-up and since LIV-SNT was not available, a final conclusion with respect to LIV could not be made. The travel and vaccination history of this animal might have shed some light on the high titre obtained despite the standard sample preparation described above.

Other flaviviruses than the ones we considered, are either not known to infect dogs, or only occur outside Europe, or depend on maintenance hosts and/or vectors currently not present in Belgium. It concerns viruses of the TBEV-serocomplex, (i.e. the mammalian group of tick-borne flaviviruses: Langat virus, Powassan virus, Kyasanur forest disease virus, Omsk haemorrhagic fever virus, Royal Farm virus, Karshi virus, Gadgets Gully virus and Alkhumra virus), as well as Dengue virus and Yellow fever virus (Dobler, 2010; Grard et al., 2007; Gritsun et al., 2003a; Gubler et al., 2007). These flaviviruses were considered irrelevant for our differential list/panel.

The TBEV seropositive dog's history revealed international travel and tick exposure, though these factors did not seem to occur simultaneously. The specific areas visited in Germany and France are not currently listed as endemic areas, though in both cases endemic areas are nearby (ISW-TBE and Baxter, 2009; RKI, 2003) and the visits took place during the tick season (Heinz, 2008). Belgium is currently considered to be non-endemic for TBEV (Donoso Mantke et al., 2008a), and since the visits to Southern Belgium occurred at the very end of the tick season (November)(Heinz, 2008), it seems less likely that the infection would have occurred there. As the dog had never been offered unpasteurized food items, the oral route of infection can be excluded.

According to the history of this dog, two possible sources of infection with TBEV can be considered. The dog could have become infected by an unnoticed tick during its travels in Europe, 5 to 7 years ago. Alternatively, it may have been infected from 2005 onwards, much closer to or even at home in West Flanders, where ticks were actually observed on the animal during the last two summers. The fipronil administration she received once a year in June is severely inadequate to ensure continuous tick protection throughout the whole tick season.

According to the available literature "TBEV seroneutralizing antibodies provide a lifelong protection" in both humans (Mickiene et al., 2002) and dogs (Bjöersdorff, 2002) and because the dog had traveled near to endemic areas, a travel-related infection seems plausible. However, according to (Leschnik et al., 2002), canine anti-TBEV-IgG are detectable in serum until "up to 9 months post infection", so it is possible that we detected an ELISA-borderline and RFFIT-positive animal, that was infected recently (Summer 2008) at home in Belgium. In both scenarios, further surveillance is strongly recommended as an early warning system for possible future emergence or incursions of TBEV in Belgium.

In view of these findings, it would be prudent to further validate and standardize an ELISA test for estimating prevalence of infection or exposure to TBEV in several species. Such a test would be needed to enable a data-based risk analysis for TBE in Belgium. Continued serological screening of TBEV in dogs and other domestic and wild sentinel species in all Belgian provinces is therefore advisable to gain more insight into the current situation. Such targeted veterinary serological screening of sentinel animals could contribute in a cost-effective way to a continuous public health epidemiosurveillance program for TBE(V) (Roelandt et al., 2010), in parallel with ongoing Belgian initiatives to improve epidemiosurveillance of vector-borne emerging threats to public health (Bottieau et al., 2009; Lizroth and Quoilin, 2009).

III.6 CONCLUSION

During the first serological screening for TBEV in Belgian sentinel dogs, a TBEV-seropositive dog was discovered. A commercial ELISA test was adapted for use in dogs and was followed by a confirmation SNT-test. Belgium is traditionally considered as non-endemic for this emerging vector-borne flavivirus. The seropositive dog had traveled to areas near to known endemic areas in 2001-2004. However, ticks were only observed on the dog in the period from 2005 onwards, when it stayed in at home in Northern Belgium. The animal has been asymptomatic throughout. Neither a travel-related infection nor the emergence of an autochthonous endemic focus can be excluded with certainty. Therefore, it is recommended to continue the screening of sentinel animals in Belgium to determine whether this dog is an isolated “case” in veterinary travel medicine, or whether it represents an early warning of emergence.

CHAPTER IV SEROLOGICAL SENTINEL SURVEY IN BELGIAN CATTLE

Adapted from: Sophie Roelandt, Vanessa suin, Flavien Riocreux, Sophie Lamoral, Sara Van der Heyden, Yves Van der Stede, Bénédicte Lambrecht, Brigitte Caij, Bernard Brochier, Stefan Roels, and Steven Van Gucht.

AUTOCHTHONOUS TICK-BORNE ENCEPHALITIS VIRUS (TBEV)-SERO-POSITIVE CATTLE IN BELGIUM: A RISK-BASED TARGETED SEROLOGICAL SURVEY.

Vector-borne and Zoonotic Diseases 2014; 14(9): 640-7.

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And adapted from: Sophie Roelandt, Flavien Riocreux, Sara Van der Heyden, Ann-Brigitte Caij, Bénédicte Lambrecht, Stefan Roels, Yves Van der Stede;

Vanessa Suin, Sophie Lamoral, Bernard Brochier, Steven Van Gucht

TYPE II EVALUATION OF COMMERCIAL DIAGNOSTIC REAGENTS: USE OF RFFIT (SNT) AND FSME "All Species" - ANTIBODY ELISA® DURING A RISK-BASED SCREENING FOR TICK-BORNE ENCEPHALITIS (TBE) AB IN BOVINE SERA

Report submitted to FAVV-AFSCA-FASFC on 13/09/2013

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IV.1 ABSTRACT

The risk of TBEV-introduction into Belgium remains high and the presence of infected wildlife in Belgium is suspected. Domestic animals can serve as excellent sentinels for TBEV-surveillance in order to install an early warning surveillance component for this emerging zoonotic disease of public health importance. In a targeted, risk-based and cross-sectional sampling design, serological screening was performed on Belgian cattle (n=650), selected from the 2010 Belgian national cattle surveillance serum bank.

All samples were subjected to a gold standard TBEV seroneutralisation test (SNT), based on the rapid fluorescent focus inhibition test (RFFIT) protocol. Seventeen bovines were seropositive (titer >1/15) and six had borderline results (1/10 < titer < 1/15). The accuracy of the SNT was confirmed in a mouse inoculation test. The overall bovine TBEV-seroprevalence in the targeted area was estimated between 2.61 and 4.29%. This confirms the presence of infected foci in Belgium for the first time. Further surveillance in cattle, other sentinels, ticks and humans at risk is recommended to further determine the location and size of endemic foci and the risk for public health.

The IgG protocol of the Progen ELISA® seemed to have an extremely low relative DSe in cattle, combined with a fairly reasonable relative DSp. The precision, predictive values, Cohen's kappa and Youden index also followed the same trends, indicating an overall low capacity of this test/protocol to distinguish and correctly classify TBEV seropositive and negative cattle. When inspecting the cattle ROC curves (AUC=54%), we felt that no great improvement could be made to this particular protocol by changing the cut-off in this species. A calculated cut-off (c = μneg + 2*SDneg) would still have led to a large amount of miss-classification.

IV.2 INTRODUCTION

Western subtype tick-borne encephalitis virus (TBEV), transmitted by *Ixodes ricinus* ticks and occasionally by unpasteurized ruminant milk, is currently the most important arthropod-borne viral infection in humans in Europe (Caini et al., 2012; ECDC, 2012; Herpe et al., 2007; Holzmann et al., 2009; Hudopisk et al., 2013; Ramelow et al., 1993). About one third of humans infected with TBEV develop neuro-invasive disease, characterized by fever and neurological signs, ranging from meningitis to severe encephalitis with or without myelitis (Haglund and Günther, 2003; Kaiser, 2008a).

These patients need hospitalisation and approximately 35-58% develop permanent sequelae ("post-encephalitic syndrome") (Haglund et al., 1996; Kaiser, 2008a). Despite low mortality rates of 0-3.9% (Donoso Mantke et al., 2008a; Süss, 2008a), Western subtype TBEV represents a risk for public health and food safety (Baumhackl, 2009; Haglund, 2002). TBE is of growing concern in several European countries (Süss et al., 1997) and about 3,000 people are hospitalized yearly (Haglund, 2002). Recently, human incidence has increased in several endemic countries (Süss, 2008a) and TBE seems to emerge in Northern/Western Europe (Donoso Mantke et al., 2008a).

TBEV seropositivity has been observed in many domestic and wild animals (Bjöersdorff, 2002; Rieger et al., 1999). Vertebrate host (sero)prevalence can be much higher than in local tick or human populations and may correlate better to the true TBEV endemicity of an area (Leschnik et al., 2002; Merino et al., 2000; Rieger et al., 1999; Süss, 2008a). Therefore, veterinary sentinel screening remains a valuable tool in identifying and characterizing endemic foci. TBEV-infected ruminants usually remain asymptomatic, but can transmit live virus in unpasteurized milk products such as cheese (Balogh et al., 2012; Kriz et al., 2009). Bovines have proved to be useful sentinels (Bjöersdorff, 2002; Leschnik et al., 2002). TBE is also emerging among Europe's canine population: its distribution and clinical incidence is increasing in Western Europe in parallel with human TBE (Beugnet and Marie, 2009; Leschnik et al., 2002; Pfeffer and Dobler, 2011). European canine TBE cases and screening studies were recently summarized by Pfeffer and Dobler (2011), while cases in European horses were summarized by Müller et al. (2006). Though these veterinary cases remain relatively rare, they are often severe with fatal outcome (Müller et al., 2006; Pfeffer and Dobler, 2011).

In Belgium, TBE is still considered an exotic disease (Donoso Mantke et al., 2008a; Süss, 2008a) and until recently, medical and veterinary TBEV surveillance was minimal (Roelandt et al., 2011; Roelandt et al., 2010). However, in 2010 a seropositive dog was detected in Belgium, though the exact origin of the infection (autochthonous or travel-related) could not be traced (Roelandt et al., 2011). Also in 2010, a National Reference Centre (NRC, WIV-ISP, Brussels, Belgium) was established to confirm the diagnosis of TBE in humans. Until now, one imported case of human TBE was diagnosed in a Belgian unvaccinated tourist returning from Austria (Brochier, personal communication, 2012). Two roe deer from south Belgium were found to be seropositive, once again indicating the possible existence of endemic foci within Belgium (Linden et al., 2012).

The aims of this study were to screen the Belgian cattle population for the presence of TBEV-specific seroneutralising antibodies. This screening was conducted following a risk-based cross-sectional design, in order to detect endemic areas in a targeted area of Belgium.

IV.3 METHODS

IV.3.1 SAMPLING AND STUDY POPULATION

In order to screen the Belgian cattle population, a study population was defined and a subset of samples was selected. The serum samples of the "TBE Targeted Population" (TBE-TP) originated from the yearly infectious disease screening (winter-)campaign for Belgian cattle (sampling frame), edition 2010. This population is assumed to be grazing cattle, since almost all Belgian cattle have outdoor access, particularly in summer. Sera were obtained by local veterinary surgeons between 01/01/2010 and 29/02/2010, a period when cattle are often stabled for winter, and submitted to regional laboratories for diagnostic testing. Samples were centrifuged and stored at 4°C at these laboratories. The selected samples were centralised by CODA-CERVA and stored at -20°C in the serum bank until TBEV testing.

The targeted geographical area was selected in a risk-based approach in order to maximize the probability of detection of any low prevalence endemic foci in a predefined "risk zone". Since TBEV displays westward expansion and is already present in known or recently emerging areas in neighbouring Germany and France: Figure IV-1 (Baxter, 2013), we selected the three most eastern Belgian provinces (Luxembourg, Liège, Limburg, Brabant Wallonne). Within each province, we prioritized all serum samples with postal codes closest to the national borders.

These provinces are currently also known as endemic for Lyme disease (*Borrelia burgdorferi*): Figure IV-2 (WIV-ISP, 2011), another vector-borne zoonotic disease transmitted by (often co-infected) *I. ricinus* ticks. In general, the most prominent clusters of both diseases largely correspond to each other, though the presence of TBEV seems mostly confined to a smaller subset of Lyme disease locations (Heinz, 2008; Süss, 2003; Zeman, 1997).

The age cohort selection was equally risk-based. Most samples (>90%) came from older cattle (>2 years), as this age category is expected to have higher titers of TBEV-specific antibodies. Indeed, older animals have spent more time on pasture and have experienced repeated tick infestation (Juceviciene et al., 2005; Sîkutova et al., 2009).

Consequentially, TBEV exposure and transmission probabilities also increase with age, resulting in higher rates of seropositivity in older animals (Juceviciene et al., 2005; Sîkutova et al., 2009). Upon detection of positive samples, additional animals from the positive herds (if available) were tested to further substantiate or refute the presence of potential endemic foci. This approach resulted in a total selection of n=650 cattle sera from 44 herds in Limburg, Liège and Luxembourg, i.e. TBE targeted population (TBE-TP; Figure IV-3).

Figure IV-1: TBEV endemic areas in the vicinity of Belgium.
Detail of FSME-Verbreitungsgebiete in Europa. Stand: mai 2013 – Baxter,2013 (www.zecken.de). Red areas: known TBEV-endemic areas – Orange areas: areas with single TBE cases

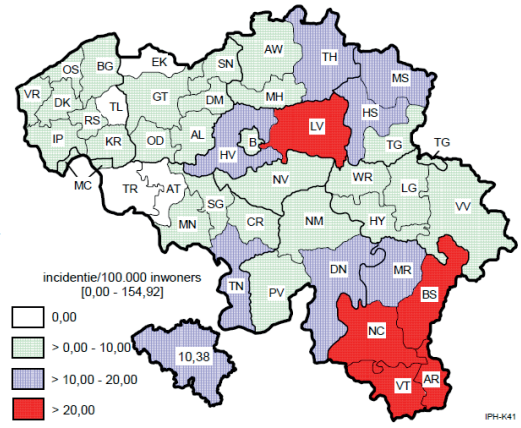

Figure IV-2: Lyme disease incidence per arrondissement.
Showing known endemic areas for Lyme disease (B. burgdorferi) in Belgium with estimates of human incidence per 100.000 inhabitants;WIV-ISP, 2011.

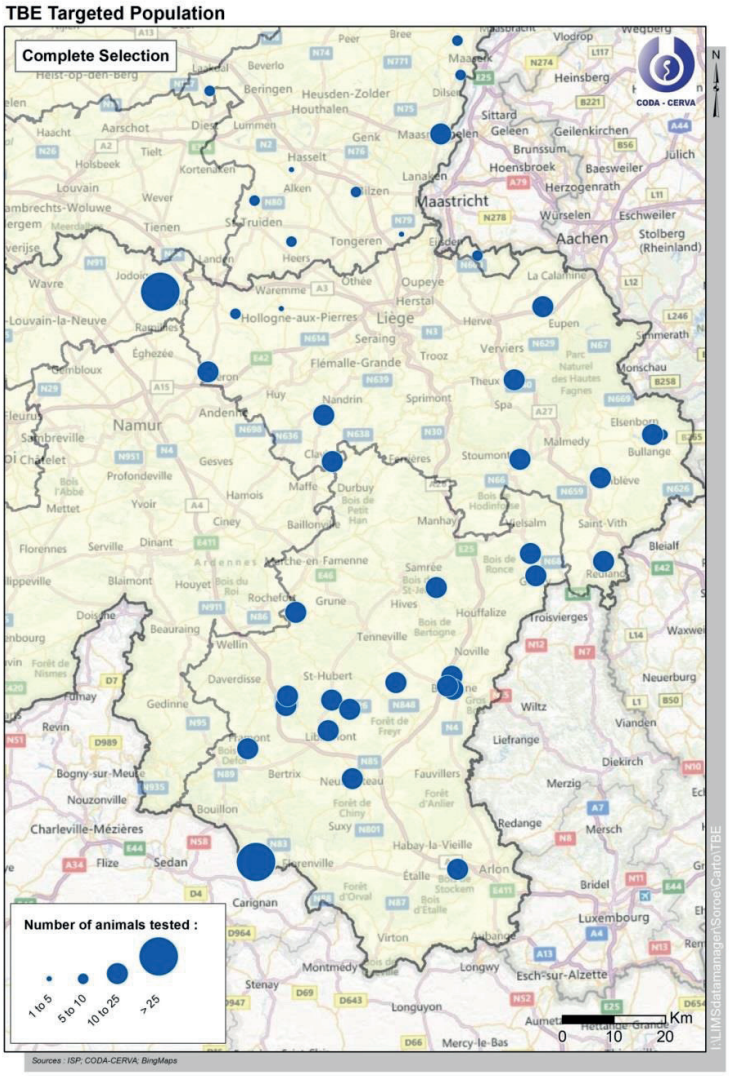

Figure IV-3: Geographical locations for the TBE targeted population (TBE-TP).
Each blue dot represents a farm (n=44); the larger the dot, the more animals of the farm were tested.

IV.3.2 DIAGNOSTIC SEROLOGY ASSAYS

IV.3.2.1 TBE virus SNT and ELISA

TBEV neutralising antibody titration in the cattle sera was performed with the SNT (rapid fluorescent focus inhibition - seroneutralisation test), which is considered a gold standard for TBE diagnosis in humans as well as in animals (Vene et al., 1998). Sera, including positive and negative controls, were diluted (1/9, 1/27, 1/81, 1/243) in 50µl Dulbecco's Modified Eagle Medium (DMEM, Gibco, The Netherlands) and supplemented with 10% inactivated fetal calf serum (DMEM-FCS, Gibco, The Netherlands) in 96-well microplates. Virus (TBEV Neudoerfl reference strain - NCPV#848) was added at a dose of approximately 1.2 log 50% endpoint tissue culture infectious doses (1.2 log $TCID_{50}$) to the wells containing the diluted sera (50µl/well).

Back titration of the challenge virus was performed in each run. A run was accepted only if the back titration yielded a virus dose between 0.8 and 1.4 log $TCID_{50}$. The virus and serum mix was allowed to incubate for 90 min at 37°C and 5% CO_2. Following this, BHK-21 cells were added to each well (35-45 × 103 cells/100 µl/well). The cells were allowed to grow for 24 h at 37°C and 5% CO_2. Then, the culture medium was discarded and the wells were washed once with 200 µl of cold PBS and once with 200 µl of cold 100% methanol. Plates were fixed with 100% methanol at 4°C for 30 min, after which the methanol was discarded and the plates were left to air-dry for 30 min at room temperature. Infected BHK-21 cells were detected by an indirect immunofluorescence staining, using a primary mouse monoclonal anti-glycoprotein E antibody (Niedrig et al., 1994) and a secondary Alexa fluor-conjugated goat anti-mouse IgG antibody (Molecular Probes). Primary and secondary antibodies were incubated at 37°C for 45 min. The plates were washed once with PBS-Tween 0.05% and twice with distilled water.

The number of foci with infected cells was counted under the fluorescence microscope. The SN titer was defined as the dilution of test serum that neutralized 50% of the virus (DIL_{50}), calculated according to the Reed & Muench method (Reed and Muench, 1938). A DIL_{50} <1/10 was considered a negative result (absence of neutralizing antibodies). A DIL_{50} between 1/10 and 1/15 was considered doubtful and a DIL_{50} >1/15 was considered positive. These cut-offs were previously selected by the TBE-NRC in the frame of the experience obtained with diagnosis in humans.

The Immunozym FSME/TBE IgG/IgM All Species-ELISA® (ELISA) (Progen Biotechnik GmbH, Heidelberg, Germany) was used previously by CODA-CERVA for the screening of Belgian dog sera. This non-competitive indirect assay uses horseradish peroxidase – Protein G conjugate to detect IgG and/or IgM against whole TBE-virus.

The kit can theoretically be used for TBEV testing in all species, including humans. In humans, this ELISA has a diagnostic sensitivity of 97% and analytical specificity of 99% for IgG (Progen Biotechnik GmbH, 2006). This ELISA also includes a borderline zone, for samples with IgG VIEU Units/Liter between 63 and 120.

In their studies, Klaus et al. (Klaus et al., 2011) used the Immunozym All-species **FSME IgM** kit in an adapted version (Müller, 1997) due to the claimed higher sensitivity of the IgG+IgM protocol, since it should additionally detect early infections. The maufacturer's IgM protocol and the IgM+IgG protocol of Klaus et al (2011) were trialled too.

IV.3.2.2 Rabies virus SNT

The possible presence of aspecific inhibitors of virus infectivity, such as complement or nonspecific contaminants, was evaluated by testing the positive sera in a rabies virus SNT, performed at the Belgian NRC of Rabies (WIV-ISP). Since Belgium has been officially rabies-free Belgium since 2001 (OIE, 2016; Van Gucht and Le Roux, 2008) besides one single imported cases in companion animals (FLI, 2015; Vaillant and teams, 2008). The surrounding countries have the same status (CDC, 2015), and since cattle are not vaccinated in Belgium, autochthonous bovine sera should not contain antibodies against rabies virus.

The principle of the rabies RFFIT-SNT is the in vitro neutralisation of 100 $TCID_{50}$ of rabies virus (CVS-11 strain: ATCC-VR959), by 4 serum dilutions (1/9, 1/27, 1/81, 1/243), in BHK-21 cells (OIE, 2013). Infected cells are detected by indirect immunofluorescent antibody staining, using a mouse anti-rabies monoclonal antibody coupled to FITC (FDI Fujibio Diagnostics). The titer is expressed in international units (IU)/ml (WHO standard)(Reed and Muench, 1938). Samples with a titer >0.5 IU/ml are considered positive.

IV.3.2.3 West Nile virus ELISA and SNT

Potential flavivirus cross-reactions with West Nile Virus antibodies were evaluated by WNV-SNT and WNV-ELISA (performed at CODA-CERVA). For the WNV-SNT, 5-fold serum dilutions were incubated in a 96-well plate with 100 $TCID_{50}$ of WNV (IS-98 STD1 strain, Genbank accession no: AF481864) for 1-1.5 hours at 37°C.

After one hour, Vero cells (CCL-81, ATCC, Molsheim, France) diluted to 1/20 were added to each well, corresponding to a seeding density per well of approximately 1.5×103 cells/cm². The plate was incubated for 3 days at 37°C with 5% CO_2. The cytopathic effect of grown virus was read by microscopy.

A commercially available inhibition ELISA kit (ID Screen West Nile Competition, IDVet®, Montpellier, France) was used to detect anti-WNV antibodies, by noting the extent of O.D. (optical density) reduction as compared to the negative control sample (N). The manufacturer's instructions were followed: sera were diluted 1/2, and optical densities (O.D.) were read at 450nm (S). For each plate, positive and negative (N) controls were included in order to allow the ELISA validation and calculation of S/N ratio for each sample (S). Sera with O.D. reduction resulting in an $S/N \leq 40$ were positive, sera with $S/N > 50$ were negative, and those having an S/N between 40 and 50 were classified as doubtful.

IV.3.2.4 Confirmatory mouse inoculation test (MIT)

A mouse inoculation test (MIT) was set up in order to confirm the presence of TBEV-specific neutralising antibodies in seropositive cattle. An intranasal inoculation model was developed in analogy with the model developed for rabies virus (Rosseels et al., 2011) and was approved by the local ethical committee of WIV-ISP (advice nr. 070130-01).

Female Swiss outbred mice (Harlan®, The Netherlands) were used at the age of 6-8 weeks. Four groups of mice (n=30) were inoculated intranasally with TBEV only (Group 1: 102.5 $TCID_{50}$), a combination of TBEV and one single negative bovine serum (Group 2: DIL_{50} neg), a combination of TBEV and one single positive bovine serum (Group 3: DIL_{50}:18-23-30) or a combination of TBEV and one single positive human serum, obtained from a vaccinated donor (Group 4: DIL_{50} >243).

Virus and serum were pre-incubated for 30 min. at 37°C, prior to intranasal inoculation. Each mouse was treated with the serum derived from one animal or person. The mice were observed daily for general and/or neurological signs, throughout the experiment until 28 days after inoculation. Scores ranged from 0 (no disease) to 7 (severe nervous disease). Symptomatic mice were euthanized by cervical dislocation when they reached a disease score ≥6.

TBEV infection in the brain was verified by qRT-PCR (Schwaiger and Cassinotti, 2003) and virus-induced lesions were examined by histopathology. Brain samples were fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 4µm and stained with haematoxylin and eosin (HE) according to standard light microscopy protocols.

IV.3.3 STATISTICAL ANALYSIS

Sample size calculations for the purpose of disease detection and freedom substantiation calculations were performed in Survey Toolbox®. The sample size that was available (n=650), was afterwards deemed sufficient for the purpose of detecting a seroprevalence of 0.55% with a confidence level of 95% (required: n=544), and assuming 100% diagnostic accuracy for SNT. Seroprevalences were calculated with 95% Wald confidence intervals (95%CI).

For the MIT, Kaplan-Meyer survival curves and log-rank tests were performed in GraphPad Prism6®; $p<0.05$ was considered statically significant. Maps were created in ArcGIS®, using the farmers' addresses/postal codes and the Belgian Lambert 1972 EPSG projection.

Test precision is defined by repeatability and reproducibility coefficients of variation, measuring intra-plate variation (CV_{repe}) and the inter-plate variation (CV_{repro}) of samples. The Common CV_{repro} calculates the overall variation, for all samples over all plates together. Commonly used precision criteria are $CV_{repe} \leq 10\%$ (15%) and $CV_{repro} \leq 15\%$ (20%) and the values can be calculated on raw OD (optical density) or on calculated (VIEU: Vienna units/Liter) values.

IV.4 RESULTS

IV.4.1 DIAGNOSTIC SEROLOGY

Based on SNT results and using the more specific cut-off (≥1/15 DIL_{50}), a total of 17 cattle (2.6% [95%CI: 1.4–3.8%]) were classified TBEV-seropositive. Additionally, 6 bovines had borderline results (1/10 < titer DIL_{50} < 1/15), which adds another 0.9% (95%CI: 0.2-1.7%) of animals with suspicious results.

The TBEV-seropositive and -doubtful sera showed no neutralisation of rabies virus (rabies virus-SNT titer <0.5 IU/ml), excluding the presence of aspecific inhibitors of virus infectivity. One TBEV-seropositive serum showed positive inhibition in the WNV-SNT (titer 1/15), whereas none of these samples showed a positive result in WNV-ELISA. However, the SNT-reactor sample was of bad quality by the time of WNV testing, which may have influenced the outcome of the WNV-SNT. Besides this one inconclusive contaminated sample, all other TBEV-seropositive samples were negative for WNV-specific antibodies. Available information on the TBEV-seropositive and -borderline animals was traced in the national cattle database.

Most bovines (n=20) were localized in Wallonia and 3 came from Flanders (Figure IV-4). All 23 animals were autochthonous (not imported), originated from 10 different herds and most were female beef cattle older than 2 years. Borderline reactors (orange) were often found in herds already containing seropositive animals (red)(Figure IV-4).

Figure IV-4: Distribution of RFFIT-SNT seropositive and doubtful bovine cases.

IV.4.2 CONFIRMATORY MOUSE INOCULATION TEST (MIT)

In the untreated mice (Groups 1-2), TBEV caused an acute lethal infection in the brain. General and neurological disease signs (rough hair coat, conjunctivitis, hunched back, isolation from the group, slowness, weakness, incoordination of movements, paralysis of the hind legs) started at 8 days post inoculation and mice needed to be euthanized due to severe neurological disease between 8-10 days post inoculation (100% mortality, median survival time 10 days).

Treatment with TBEV-seropositive sera with increasing SNT-titers (Groups 3-4: bovine or human) resulted in a delayed onset of neurological signs and a significant increase of the median survival time (Group3: 18 DIL_{50}) ($p<0.05$, Log-Rank test, Graph Pad Prism6®), or complete protection against disease (Groups 3-4: DIL_{50}: 23-243). There was a positive and statistically significant association ($p<0.05$) between the seroneutralising antibody titers and the median survival time in the MIT. For sera with a titer of 0, 18 or ≥23 DIL_{50}, median survival times were respectively 8.5, 11.5 and >28 days (Figure IV-5).

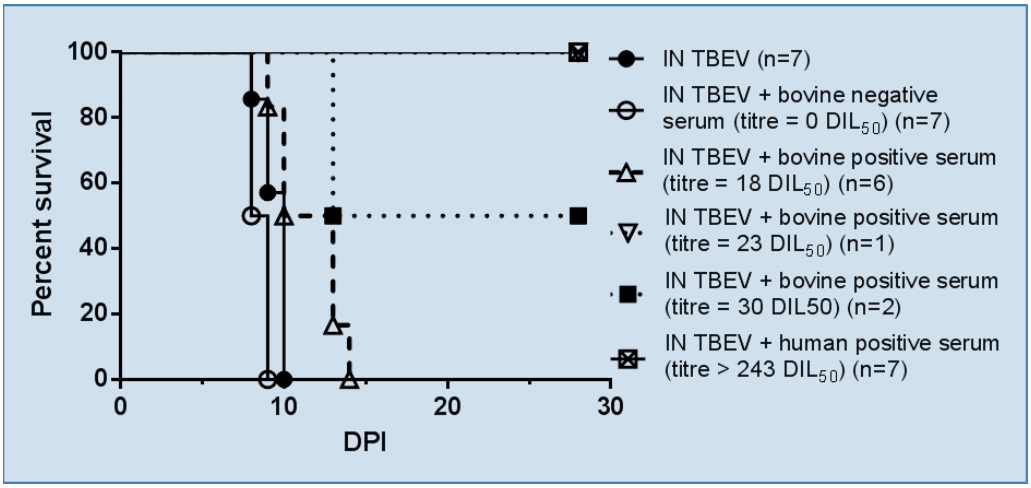

Figure IV-5: Kaplan-Meier survival curves: Neutralisation of TBEV in a mouse inoculation model.
IN: intranasal; DIL_{50}: 50% endpoint dilution titer; Median survival times were significantly prolonged in the groups treated with positive bovine sera of 18-30 DIL_{50} ($p<0.05$, Log-Rank test).

Histopathologic examination of the brains collected from the SPF mice, revealed no TBEV or virus-specific lesions were detected in the brains of non-diseased survivor mice (Groups 3-4: DIL_{50}: 23-243). A focal non-specific gliosis (Figure VI-6a) was observed both in the control animals as well as in the human and bovine serum-treated animals, but more so in the controls. The non-treated (control mice) brains were further characterized by moderate to marked non-suppurative meningo-encephalitis with mononuclear cell infiltration in the leptomeninges, multifocal (peri-)vasculitis, focal gliosis, and necrosis in the cerebral parenchyma (Figure IV-6b). This is compatible with a classical viral meningo-encephalitis, as described also for natural TBEV-infection. TBE-virus (qRT-PCR) and clear virus-induced histopathological lesions were detected only in the brains of all TBEV-infected control mice upon euthanasia (Groups 1-3: DIL_{50}: neg-18).

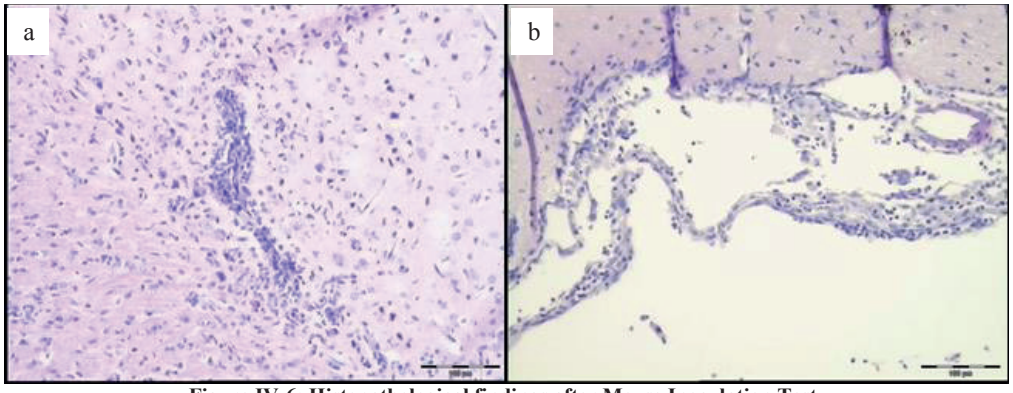

Figure IV-6: Histopathological findings after Mouse Inoculation Test.
a: Gliosis and (peri-)vasculitis in the cerebrum of TBE infected non-treated mice;
b: Non-suppurative meningo-encefalitis in TBE infected non-treated mice.

IV.4.3 Evaluation of Progen ELISA

First, we present the cross-classified binary test results for all samples in Table VI-1. Both tests include a zone in which results are considered to be “borderline” (syn. non-interpretable, doubtful, grey zone). Samples with SNT titers between 1/10 and 1/15 were considered borderline, whereas for the ELISA it concerned samples with IgG VIEU units/ml between 63 and 126. Using SNT results as gold standard results, a total of 17 cattle were found to be TBEV-seropositive by SNT, and a further 6 cattle had borderline SNT results (Table IV-1).

Table IV-1: Complete selection TBE-TP.				
IgG ELISA	*SNT*			
	pos	border	neg	Total
pos	1	0	15	16
border	2	0	52	54
neg	14	6	560	580
Total	**17**	**6**	627	650

Table IV-1: Cross-classified test results for Complete selection of TBE screening population (n=650).
IgG ELISA: FSME All Species ELISA IgG protocol; RFFIT: rapid fluorescent focus inhibition test; border(line): ELISA VIEU between 63 < x < 126 or RFFIT titer 1/10 < x < 1/15

The calculated precision CV's are represented in Tables IV-2. The classic accuracy parameters are summarized with 95% confidence intervals (Wald) in Table IV-3. The AUC from the ROC curves (data not shown) were approximately 54% for both SNT cut-offs.

Table IV-2: IgG ELISA Repeatability. *Variation per sample per plate; CVrepe = STDEV / AVG * 100; Criterium: <15%*								
Sample	*day 1 OD*	*day 1 VIEU*	*day 2 OD*	*day 2 VIEU*	*day 3 OD*	*day 3 VIEU*	*day 4 OD*	*day 4 VIEU*
A10108-1436(B)	28.46%	16.63%	7.77%	7.11%	7.25%	11.62%	20.18%	19.08%
A10108-1436	10.24%	6.32%	6.72%	6.16%	5.28%	8.94%	16.51%	15.57%
212-192246(B)	20.08%	11.11%	6.79%	6.24%	4.43%	7.11%	1.19%	1.15%
212-192246	14.52%	8.25%	4.85%	4.44%	5.03%	8.11%	10.41%	9.93%
SF	/	/	7.13%	6.53%	3.14%	5.30%	/	/

Table IV-2: ELISA Repeatability.
CV_{repe}: repeatability coefficient of variation; STDEV: standard deviation; AVG: mean; OD: optical density; VIEU: Vienna Units/Liter; Red values: failing criterion.

Table IV-3: IgG ELISA Reproducibility. *Variation per sample over all plates.*		
Sample	*CV-OD*	*CV-VIEU*
A10108-1436(B)	26.21%	28.16%
A10108-1436	26.51%	32.50%
212-192246(B)	10.89%	26.05%
212-192246	3.45%	23.42%
FS Foetal Serum	4.90%	32.94%
COMMON REPRO	18.59%	30.45%

Table IV-3: ELISA Reproducibility.
*Within plate variation for 5 fetal calf sera in ELISA; CVrepro: reproducibility coefficient of variation, CV repro = STDEV / AVG * 100 and Criterium: <20%; STDEV: standard deviation; AVG: mean; OD: optical density; VIEU: Vienna Units/Liter; Red values: failing criterion.*

Table IV-4: IgG ELISA Accuracy Characteristics				
Scenario	*Parameter*	*Estimate*	*95% Confidence Interval*	
			Lower limit	Upper limit
Border = pos: ELISA vieu >63 SNT > 1/10	D Se	13.043%	0.000%	26.807%
	D Sp	89.314%	86.896%	91.732%
	PPV	4.286%	0.000%	9.030%
	NPV	96.552%	95.067%	98.037%
	Youden	0.024	-0.116	0.163
	Kappa	0.012	-0.053	0.033
	App Prev	10.769%	8.386%	13.152%
	True Prev	4.286%	2.118%	4.959%
Border = neg: ELISA vieu >120 SNT > 1/15	D Se	5.882%	0.000%	17.068%
	D Sp	97.630%	96.445%	98.815%
	PPV	6.250%	0.000%	18.111%
	NPV	97.476%	96.255%	98.697%
	Youden	0.035	-0.077	0.148
	Kappa	0.036	-0.041	0.113
	App Prev	2.462%	1.270%	3.653%
	True Prev	2.615%	1.388%	3.842%
Border = mix: ELISA vieu >63 SNT > 1/15	D Se	17.647%	0.000%	20.578%
	D Sp	89.415%	87.019%	91.812%
	PPV	4.286%	0.000%	9.030%
	NPV	97.586%	96.337%	98.835%
	Youden	0.071	-0.112	0.253
	Kappa	0.028	-0.031	0.087
	App Prev	10.769%	8.386%	13.152%
	True Prev	2.615%	1.388%	3.842%

Table IV-4: IgG ELISA Accuracy Characteristics.
Calculated results for parameters of interest under three different borderline scenarios. IgG ELISA: FSME All Species ELISA IgG protocol; SNT: rapid fluorescent focus inhibition test; possible cut-offs ELISA: VIEU 63 - VIEU 126 ; possible cut-offs SNT: titer 1/10 - titer 1/15; D Se: diagnostic sensitivity; D Sp: diagnostic specificity; PPV: positive predictive value; NPV: negative predictive value; Youden: youden index = D Se + D Sp – 1; Kappa: coefficient of test agreement; app prev: apparent prevalence; true prev: true prevalence.

The IgM+IgG protocol (veterinary units U/L) was also tested with a subset of our cattle sera (n=20 SNT positives/doubtfuls and n=23 negatives). Bearing in mind the low number of available cattle positives, this did not seem to result in improved sensitivity when compared to the SNT: DSe 15% – DSp: 100%). Only 3 samples of this small positive panel showed any IgG+IgM reaction (>5 U/L). When tested inthe regular IgM protocol after kaolin treatment, 12 SNT- positive and 6 SNT-borderline samples all remained negative in the IgM protocol (<40 VIEU/ml).

IV.5 DISCUSSION

IV.5.1 ELISA ACCURACY AND PRECISION

Regardless of the borderline/cut-off scenario used, the IgG protocol of the FSME All Species ELISA®, when used with the kit calibrator samples (human origin), seems to have an extremely low relative DSe in cattle, combined with a fairly reasonable relative DSp. The predictive values and Youden index also follow the same trends, indicating an overall low capacity of this test/protocol to distinguish and correctly classify TBEV seropositive and negative cattle (as opposed to results in other species). Cohen's Kappa (Landis and Koch, 1977) equally indicates very poor agreement and high discordance between the IgG ELISA and the SNT results.

The IgM protocols did not improve this at all, thereby supporting our initial hypothesis that IgG-testing for previous exposure is potentially more likely to have a higher overall sensitivity in field studies, at the inevitable cost of some false positives.

When evaluating the ELISA precision, we observe that the fetal calf sera show considerable variation, both in OD and VIEU calculations values. This variation is larger than the amount that would be allowed in official disease surveillance and control programs, even with relaxed criteria (15% and 20%).

This ELISA test was intended as a sensitive screening test in multiple species, however it was until now mostly validated in humans and dogs. We remarked previously that the cut-off for the IgG protocol could possibly be lowered to evaluate canine sera (Roelandt et al., 2010). However, when inspecting the cattle ROC curves (AUC=54-59%; Figure IV-7), we felt that no great improvement could be made to this particular protocol by changing the cut-off, since the total area under the curve is very close to 50%. In order to reach a DSe of 88%, one would have to accept a DSp of 16%, which might only work in a very low prevalence setting.

A calculated cut-off, based on negative animals is another option: $c = \mu neg + 2*SDneg$. However, with $\mu neg = 38.90$ and $SDneg = 29.58$, then $c = 98$ VIEU. When we use the fetal calf serum results as an alternative negative group, $\mu neg = 11.427$ and $SDneg = 3.480$, and in this case $c = 18.387$ VIEU. These calculated cut-offs, though quite different, would both still have led to a large amount of miss-classification, since the distributions of the SNT-positive and SNT-negative cattle greatly overlap, as illustrated in Figure IV-8 (purple and yellow lines). It is questionable whether a larger sample size would change this.

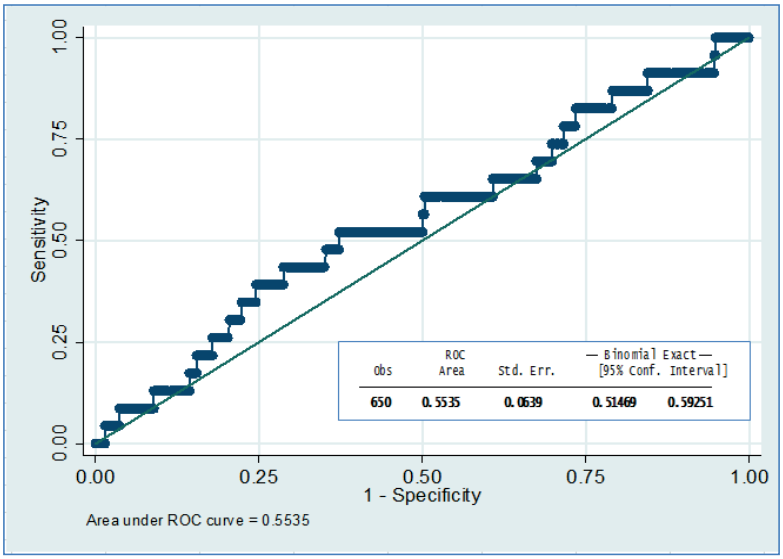

Figure IV-7: ROC curve for IgG ELISA with SNT borderlines counted as positive.

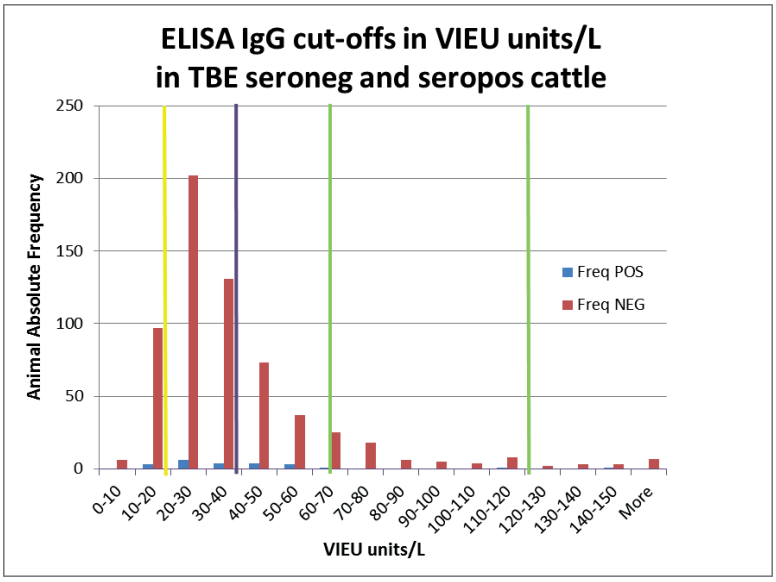

Figure IV-8: Possible ELISA cut-offs in relation with cattle test results.
Green: manufacturer's cut-off ; Purple: neg. cattle cut-off; Yellow: Fetal calf serum cut-off

IV.5.2 INTERPRETATION OF SNT RESULTS

In analogy to human TBE diagnostics (Holzmann, 2003; Rendi-Wagner, 2004), seroneutralisation tests are the gold standard for serology in animals, since they are highly specific and not affected by cross-reactive antibodies against other flaviviruses (Klimeš et al., 2001; Leschnik et al., 2002; Reiner and Fischer, 1998; Vene et al., 1998). By SNT testing, a TBEV-seroprevalence of 2.6-3.5% was demonstrated in autochthonous Belgian cattle in the targeted risk zone in 2010. This is an important finding, since Belgium was until now considered to be TBEV-free.

The specificity of the SNT results was confirmed by the absence of reactivity against other viruses (rabies or West Nile) in SNT- or ELISA-tests and by the protection provided by the TBEV-seropositive bovine sera in the mouse inoculation test (MIT), which was SNT-titer dependent. The WNV inhibition in one contaminated sample might have been due to an unspecific contamination of that particular serum.

The relatively older age profile of the reactor cattle corresponds with the targeted sampling design, but also with data in the literature: older animals are expected to show higher rates of seropositivity due to prolonged tick and TBEV exposure while grazing on pasture (Juceviciene et al., 2005; Sîkutova et al., 2009). Additionally, Juceviciene et al. (2005) comment that seroprevalence and titers in cattle may fluctuate with the (tick) season and that values are lowest in winter. Consequently, summer TBEV-seroprevalence in cattle from the targeted areas may be higher than what could be concluded from these samples, obtained in winter.

For any design prevalence (0.1-50%), the observed number of positive reactors (17 or 23 depending on the cut-off) was always too high to substantiate freedom of TBEV for the targeted TBE-TP population (Survey Toolbox®, p-values>0.05). Thus, freedom can no longer be substantiated in the targeted Belgian provinces. However, the 2010 wintertime TBEV-seroprevalence can reasonably be expected to be lower than 4.0% (if 17 reactors, p-value = 0.009) or 5.0% (if 23 reactors, p-value <0.047).

In the available European literature, cattle TBEV-seroprevalence in known TBEV-endemic foci usually fluctuates around 2-3% (Cisak et al., 2010; Juceviciene et al., 2005; Sîkutova et al., 2009) and can be as high as 36-91% in the most severely affected areas (Brummer-Korvenkontio et al., 1973; Ernek and Kozuch, 1970; Süss, 2008a). Based on this information, it appears that there is now evidence that south-east Belgium indeed contains TBEV-endemic foci.

Up to October 2013, no locally acquired human case of TBE has been confirmed in Belgium. The number of human infections might indeed be low, but we cannot exclude some degree of underreporting or misdiagnosis of probable cases, due to the generally poor TBE-awareness in Belgian health care practitioners. In addition, about two thirds of human infections remain asymptomatic or induce only aspecific fever symptoms, which may easily go by undiagnosed in a low-prevalence country.

As expected, we found some locations of TBEV-seropositive cattle (Figure IV-4) within known Belgian Lyme disease hot spots (Figure IV-4)(WIV-ISP, 2011), and where we would first expect TBEV-incursion from abroad due to vicinity to known endemic foci in the neighboring countries (Figure IV-1). These areas are also clearly suitable to support sylvatic virus circulation in local wildlife, due to favorable tick habitat, available hosts and climate (Roelandt et al., 2010). The prevalence of TBEV outside of the targeted areas remains to be studied.

This study was based on a risk-based cross-sectional approach, in order to be able to detect TBEV - if present - in Belgium. Hence, sample selection was targeted and biased towards certain provinces (south-east) and municipalities (border) and towards cattle of an older age category. This design is also reflected in the fact that most of the seropositive cattle seem to be located in Wallonia. To obtain a more unbiased estimate of the true TBEV-seroprevalence in Belgian cattle and of local endemic foci, a future screening may follow a random stratified sampling with larger sample size. In Belgium, the yearly official cattle serology campaign remains an excellent source of such randomly selected samples and it would suffice to simply adapt the selection process to this end.

Continued serological screening for TBEV in cattle, dogs and other domestic and wild sentinel species in all Belgian provinces is advisable, in order to gain more insight into the current situation and to better define the localization of endemic foci. Such a veterinary serosurveillance tool would contribute in a cost-effective way to target the TBEV surveillance program in humans and public health preventive actions.

IV.6 CONCLUSION

In a risk-based cross-sectional approach, a seroprevalence of 2.6-3.5% was found in older cattle located in targeted Belgian provinces. Seropositive cattle were clustered in a few municipalities, demonstrating for the first time the presence of TBEV-infected foci in the targeted areas in south-east Belgium.

The IgG protocol of the Progen ELISA® seemed to have an extremely low relative DSe in cattle, combined with a fairly reasonable relative DSp. The precision, predictive values, Cohen's kappa and Youden index also followed the same trends, indicating an overall low capacity of this test/protocol to distinguish and correctly classify TBEV seropositive and negative cattle. When inspecting the cattle ROC curves (AUC=54%), we felt that no great improvement could be made to this particular protocol by changing the cut-off in this species. A calculated cut-off ($c = \mu neg + 2*SDneg$) would both still have led to a large amount of miss-classification.

In conclusion, Belgium should no longer be considered "free" of TBEV. Given the relevance of TBEV for the food chain through consumption of unpasteurized milk and cheese and through its considerable public health burden in other European countries, the finding of TBEV-seropositive cattle in Belgium warrants further surveillance and follow-up, both by veterinary and public health officials and clinicians.

CHAPTER V SEROLOGICAL SENTINEL SURVEY IN FLEMISH WILD BOAR

Adapted from: Sophie Roelandt, Vanessa Suin, Yves Van der Stede, Sophie Lamoral, Sylvie Marche, Marylène Tignon, Juan Carlos Saiz, Estela Escribano-Romero, Jim Casaer, Bernard Brochier, Steven Van Gucht, Stefan Roels, and Muriel Vervaeke.

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V.1 ABSTRACT

The risk of TBEV-introduction into Belgium remains high and the presence of seroconverted wildlife and domestic animals in Belgium has already been demonstrated in multiple studies. In the frame of a Flemish wildlife surveillance in 2013, a serological screening was performed on sera from Flemish wild boar (*Sus scrofa*; n=238) in order to detect TBEV-specific antibodies. These sera were taken in 2013 throughout the whole Flemish wild boar population range.

All samples were subjected to gold standard TBEV seroneutralisation (SNT). Seven wild boar were seropositive and showed moderate to high SNT-titers - three had borderline results. Seroprevalence was estimated around 4.20% (95%CI: 1.65-6.75%). Other Flaviviridae (Classical Swine Fever, West Nile Fever, Louping Ill viruses) were ruled out and thirteen available tonsils tested negative in TBEV RT-PCR.

The test characteristics of a commercially available TBEV-ELISA were assessed against the gold standard results. Using the manufacturer's cut-offs and an alternately positive/negative interpretation of SNT-borderline results, the IgG protocol of this ELISA showed low diagnostic sensitivity and good diagnostic specificity (DSe: 40-57% and DSp: 91-92%). ELISA agreement with the SNT was judged "slight to fair". ROC-analysis showed that for early detection screening purposes (with SNT follow-up), the ELISA cut-off might be placed as low as 35 Vienna-units: this would result in improved DSe (70-71%) at the cost of DSp (64.04-69.74%).

This study showed the presence of TBEV-specific antibodies in wild boar and potential TBEV-foci in Flanders. Ongoing wild boar surveillance could serve as sentinel warning system for public/human health prevention. Additional active surveillance and direct testing are now recommended to attempt virus detection and to further determine the characteristics of endemic foci, while continued passive medical and veterinary surveillance is indicated to monitor the potential risk for Belgian public health.

V.2 INTRODUCTION

Tick-borne encephalitis remains a significant public health concern wherever Ixodid tick vectors are present: thousands of medical cases are reported annually throughout Eurasia. The Western/European subtype of TBEV first causes an influenza-like illness, followed in 30% of cases by serious neurological disease 4-10 days post-recovery. TBE may result in death or long-term neurologic/neuropsychiatric sequelae (CDC, 2014b; ECDC, 2012; WHO, 2011, 2014a, b).

Though veterinary cases remain relatively rare, they are often fatal (Müller et al., 2006; Pfeffer and Dobler, 2011). There exists an international consensus that veterinary surveillance (ECDC, 2012; Süss, 2011) may provide superior information on the true eco-epidemiological situation than medical TBEV-surveillance, especially in low prevalence areas (ECDC, 2012; Klaus et al., 2011; Klaus et al., 2010a; Süss, 2011).

While small rodents are TBEV-reservoirs, larger wild/domestic mammalian hosts will support TBE-virus indirectly by amplifying ticks (ECDC, 2012; Gritsun et al., 2003b; Süss, 2011). These hosts can be used as sentinels: TBEV-seropositivity was indeed observed in Eurasian wild boar (*Sus scrofa*) (Balling et al., 2014; Borcic et al., 1990; Cisak et al., 2012; Hubalek et al., 1993; Juricova, 1992; Kriz et al., 2014; Zeman and Januska, 1999). This seroprevalence may be higher than in other hosts (Cisak et al., 2012; Gómez-Martínez, 2014) and wild boar may be a risk factor for human exposure (Pugliese et al., 2007; Pugliese et al., 2002). At sufficient sample sizes wild boar studies may allow spatial interpretations at municipality level (Cisak et al., 2012).

In Belgium, TBE was until recently considered exotic (Donoso Mantke et al., 2008a; ECDC, 2012; Süss, 2008a). Until 2014, five imported cases of human TBE from Scandinavia, Austria and Kyrgyzstan were detected at the Belgian National Reference Centre (WIV-ISP, Brussels, Belgium). Meanwhile, serological evidence was collected from veterinary surveys in dogs (Roelandt et al., 2011), roe deer (Linden et al., 2012; Tavernier et al., 2015) and cattle (Roelandt et al., 2014).

The Flemish wild boar population (northern Belgium) is steadily increasing in numbers/range (Scheppers et al., 2011; Scheppers et al., 2013), as are many European populations (Apollonio et al., 2010). Its potential density was estimated based on natural resources present in proposed wild boar management zones: Figure V-1a (Scheppers et al., 2011). Moreover, data on hunting bags per community were used to determine relative high density zones: Figure V-1b (Scheppers et al., 2013, 2014; Vervaeke, 2012).

The aims in the following survey were: (1) screening a representative sample of Flemish wild boar for TBEV-specific seroneutralising antibodies; (2) detecting potential TBEV-endemic areas in wild boar habitat; (3) evaluating accuracy of a commercial veterinary ELISA.

V.3 MATERIALS AND METHODS

V.3.1 STUDY POPULATION AND SAMPLING

During 2013, Flemish wild boars were primarily reported/shot in the province of Limburg and secondarily in West-Flanders, Antwerp and East-Flanders: see Figure V-1 (Scheppers et al., 2014). Population size was estimated at roughly 1,000-3,000 heads (Vervaeke, pers.comm.). TBEV-foci are often located in subsets of Lyme disease locations (Heinz, 2008; Randolph and Sumilo, 2007; Süss, 2003; Zeman, 1997), such as Limburg (WIV-ISP, 2011).

Since 2011, surveillance is performed in this hunted wild boar population for Aujeszky's disease, Classical Swine Fever virus and Brucellosis (Vervaeke, 2012). Blood was aspirated from the heart or large blood vessels straight after the kill (Vervaeke, 2012). The samples were identified, cooled and transported to the regional laboratory DGZ and the National Reference Laboratories at CODA-CERVA for veterinary testing and were stored afterwards at -20°C.

Our study population (Figure V-2) was defined as 238 sera from hunted animals from the 2013 surveillance in Limburg and Antwerp (n=153+8) and West Flanders (n=77). The samples were obtained from 24 official municipality NIS-codes (Statbel) (Figure V-2) and contained roughly 33% females, 33% males and 33% unknowns. As many samples as possible were retained from the whole geographic range from that year's hunting bag (n=628) (Scheppers et al., 2014). This was to increase sample size and detection probability, and to avoid sampling bias, i.e. to find TBEV seropositivity only in a pre-defined "risk zone" in the eastern parts of Belgium, as in (Roelandt et al., 2014). As such, it would be possible to draw conclusions about the whole Flemish wild boar range, instead of extrapolating.

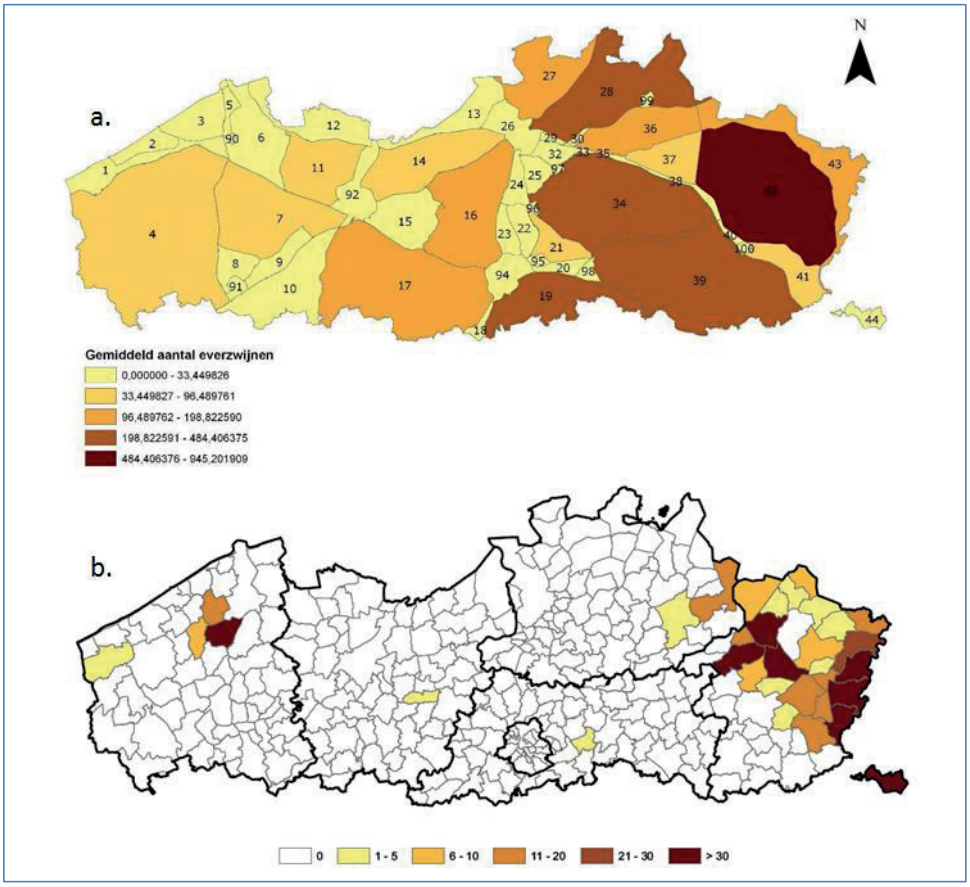

Figure V-1: Flemish Wild boar population estimates.
1a. Numbers estimated based on natural resources, per management zone (Scheppers et al., 2011);
1b. Numbers reported by the public, per municipality (Scheppers et al., 2014)

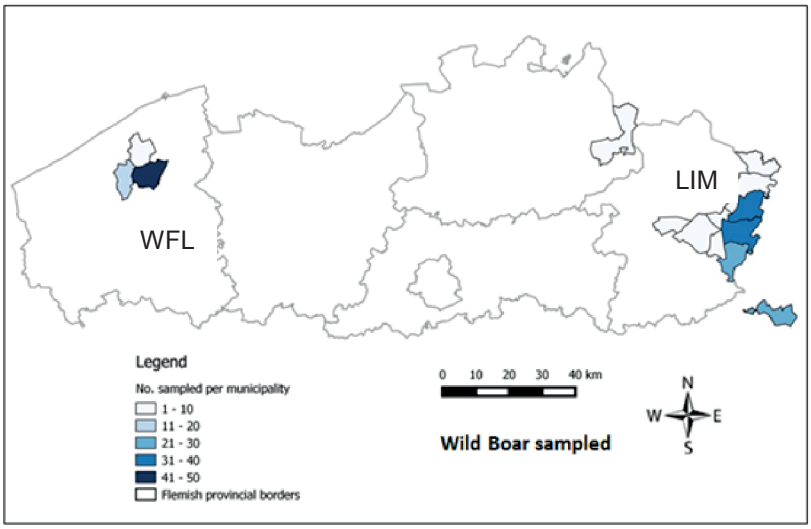

Figure V-2: The study population (n=238).
LIM: Limburg+Antwerp provinces; WFL: West Flanders province

V.3.2 DIAGNOSTIC ASSAYS

V.3.2.1 TBEV testing

a. Serology

Neutralising antibodies were titrated in kaolin-treated wild boar sera (Van der Stede et al., 2003) Radda et al., 1968) with the RFFIT-SNT (rapid fluorescent focus inhibition seroneutralisation test), considered a gold standard for medical/veterinary diagnosis (Vene et al., 1998). The test was performed by WIV-ISP, as described in (Roelandt et al., 2014). Virus neutralization was measured by assessing reduction in microscopic fluorescent TBEV-foci. Two cut-offs were used to classify results (<1/10: negative; 1/10-1/15: borderline; >1/15: positive).

A commercially available ELISA (Immunozym FSME/TBE IgG All-Species-ELISA®, Progen Biotechnik GmbH, Heidelberg, Germany) was used to detect TBEV- specific IgG-antibodies in the sera. This non-competitive indirect assay uses horseradish peroxidase – Protein-G conjugate to detect IgG against whole TBE-virus.

The kit can theoretically be used for TBEV testing in all species, including humans. However, it was only validated for humans, where the IgG-protocol has diagnostic sensitivity (DSe) of 97% and analytical specificity of 99% (Progen, 2006). It was previously used as screening test in foxes (Rieger et al., 1999; Wurm et al., 2000) and wild boar (Cisak et al., 2012). We followed the manufacturer's instructions (Progen, 2014): sera were diluted 1/50 and optical densities were read at 450-620nm (reference 620-690nm). Standard curves were generated with five kit calibrator samples. Sample concentrations were read from these in standardized Vienna Units (VIEU/ml; <63: negative - 63-126: borderline - >126: positive).

b. RT-PCR

There was not enough serum volume left to allow qRT-PCR testing. Instead, homogenized tonsillar tissue (in PBS) was recovered at CODA-CERVA, for thirteen animals from the original study population. These samples were tested at WIV-ISP using an in-house TBEV-specific qRT-PCR protocol based on Schwaiger and Cassinotti's (Schwaiger and Cassinotti, 2003). Tonsils were homogenized in lysis buffer (from Rneasy mini kit, Qiagen) by using lysis kit stainless beads (0.9-2 mm) (Next advance technology).

A 200 µl of homogenate was extracted by using the Rneasy mini kit from Qiagen. Reverse transcription was performed using qScript cDNA SuperMix (Quanta BioSciences). 18 µl of RNA was added to a final volume of 20 µl with 5 × reaction buffer qScript supermix (Quanta BioSciences,). This mixture was incubated for 5 min at 25°C, for 30 min at 42°C and finally for 5min at 85 ◦C. The qPCR amplification was performed on an MXPro3000 from Stratagene. The reaction mix consisted of 12.5 µL of 2 x qPCR Supermix (Quanta BioSciences), 5 μL of RT product, 0.4 µM of each TBEV primers and probe and 0.4 µM of each 18RS primers and 0.08 µM probe.

Cellular ribosomal 18RS RNA was used as an extraction control. Primers and probe used for amplification were synthesized by IDT. All samples were analyzed in duplicate. The amplification program consisted of 2 min at 95°C for initial denaturation followed by 45 cycles of 15s at 95°C, 30s at 55°C and 30s at 72°C. Real-time PCR performed on an MxPro3000 real-time PCR system (Stategene). All samples were analyzed in duplicate on MxPro3000 real-time PCR system (Stategene).

V.3.2.2 Cross-reactivity testing

A commercial indirect immunofluorescence test (IFA; Biochip Flavivirus-Mosaic-3, Euroimmun®, Germany) was adapted to detect porcine antibodies. The slides are coated with virus-infected cells: TBEV, West Nile (WNV), dengue (DENV types 1-4), Japanese encephalitis (JEV) or yellow fever (YFV). The manufacturer's instructions were followed, using diluted sera (1/10, 1/100, 1/1000). Virus-specific antibodies were detected by secondary goat anti-swine antibody (1/1000, AbD Serotec®) and tertiary FITC-labelled rabbit anti-goat antibody (1/500, Molecular Probes®). Read-out was performed under an Olympus® fluorescence microscope (Figure V-3a-c).

Louping Ill was investigated by haemagglutination inhibition (LIV-HIT; Clarke and Casals,1958) at Moredun Scientific Research Institute (Scotland, UK), modified to microtitre plate version with cut-off 1/20, after heat inactivation (1h/65°C) and absorption (kaolin - goose erythrocytes).

In-house WNV-SNT and USUV-SNT tests were performed by Instituto Nacional de Investigación y Tecnologia Agraria y Alimentaria (INIA, Spain), using susceptible Vero cells and 2-fold sample dilutions from 1/20 onwards, after heat inactivation (1h/65°C) (Alonso-Padilla et al., 2009; Escribano-Romero et al., 2015).

CSFV-E2 antibodies were investigated with HerdChek CSFV-ELISA (IDEXX, The Netherlands) with values expressed as blocking percentages (30-40%: doubtful - >40%: positive). Neutralizing response was determined according to the OIE Manual (Anonymous, 2004) using the Alfort/187 strain. The 50%-neutralising doses (ND_{50}) were expressed as the last dilution giving virus neutralization (≥10: positive).

V.3.2.3 Statistical Analysis

Sample size calculations for disease detection were performed in Survey Toolbox® AusVet1.04 (Cameron, 1999) and WinEpiscope®2.0 (Thrusfield et al., 2001). We assumed 100% diagnostic accuracy for SNT and total population size of 3,000. Our obtained sample size (n=238) was afterwards deemed sufficient for the purpose of detecting a seroprevalence of 1.25% with 95% confidence and 5% error (required: n=227).

Provincial sample sizes for Limburg+Antwerp (actual: n=161; estimated: N~2,200) and West Flanders (actual: n=77; estimated: N~800) were suitable to detect design prevalences of 1.80% (required: n=159) and 3.50% (required: n=77) respectively. Probability of freedom despite an observed number of SNT-reactors was investigated with design prevalence ranging between 0.1-10% and with SNT as gold standard (100% DSe/DSp; Survey Toolbox®).

ELISA accuracy evaluation was performed in R (CoreTeamR, 2013), using package epiR (Stevenson et al., 2014), with Fleiss kappa's (Fleiss et al., 2003) and post-test disease probabilities (Hunink and Glasziou, 2001). Package pROC was used to construct a non-parametric ROC-curve (receiver operating characteristic) (Robin et al., 2011), with DeLong 95%CI for area under the curve [AUC] (DeLong et al., 1988).

SNT-seroprevalences and confidence intervals according to sample size, (Wald: n>100, Agresti-Coul: 30<n<100, Exact-Binomial: n<30; (Brown et al., 2001) were calculated using Prevalence package (Devleesschauwer et al., 2013).

The study population (Figure V-2) and its seroprevalence (Figure V-5) were mapped using QGIS®2.2-Valmiera (DevelopmentTeam, 2014), using a Flanders vector layer in Belgian Lambert 1972 EPSG-projection.

V.4 RESULTS

V.4.1 DIAGNOSTIC TEST RESULTS

TBEV-serology results were cross-classified (Table V-1): 11 boars tested ELISA-seropositive and 14 were classed as borderline. Based on RFFIT-SNT results using the more specific cut-off (>1/15), seven boars (2.9%; 95%CI: 0.79-5.09%) were classified TBEV-seropositive, including three ELISA-negatives. Four seropositives presented with moderate (1/17-1/25-1/33-1/47) and three with high SNT-titers (1/127-1/164-1/243). Three animals were SNT-borderline (1/12-1/10-1/14), adding another 1.26% (95%CI: 0.00-2.68%) of reactors. Fourteen ELISA-borderlines and most (7/11) ELISA positives were found SNT-negative. TBE-virus was not detected in any of the 13 tonsils by qRT-PCR.

Table V-1: Cross-classified TBEV serology results

ELISA	SNT			
	pos	borderline	neg/NI	Total
pos	4	0	7	11
borderline	0	0	14	14
neg	3	3	207	213
Total	7	3	228	238

Table V-1 : **Cross-classified TBEV serology results.**
ELISA: Immunozym FSME/TBE IgG All Species-ELISA® ; SNT: seroneutralisation test, i.e. reduction of fluorescent focus inhibition test RFFIT; NI: non-interpretable SNT, considered to be negative.

The results of the cross-reactivity tests for 10 TBEV-reactor samples (SNT-positive/doubtful and/or ELISA-positive) are summarized in Table V-2. Six of these samples showed borderline (1/20) or positive (1/40-1/80) reactions in the LIV-HIT. Sample no.8 tested positive in WNV/USUV-SNT and LIV-HIT. Two samples reacted in CSF-ELISA, but this was not confirmed in CSF-SNT and they were considered negative. Highly TBEV-SNT/ELISA-positive samples (no.2,3,4) reacted TBEV-IFA positive and negative for the other flaviviruses (WNV/JEV/YFV/DENV1-4), while low SNT-positives (no.1,9,10) and SNT-negatives/ELISA-positives (no. 5,6,7) did not (Figure V-3a-c).

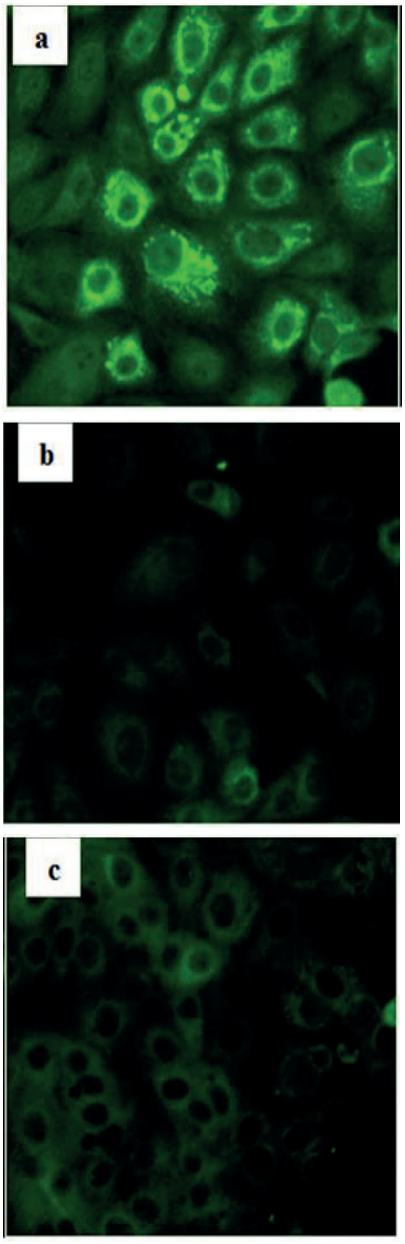

Figure V-3a-c: Immunofluorescence Assay (IFA) images at 20x magnification.
3a: TBEV seropositive sample results in granular fluorescence in the cytoplasm of TBEV-infected cells;
3b: TBEV seronegative sample gives no staining of TBEV-infected cells;
3c: TBEV-seropositive sample gives no staining of West Nile virus-infected cells

Table V-2: Cross-Reactivity Results of the Confirmation Panel (TBEV-reactors)											
Sample	**TBEV IgG ELISA (VIEU/ml)**	**TBEV SNT (titer)**	**TBEV IFA (titer)**	**LIV HIT (titer)**	**CSFV ELISA/SNT**	**USUV 90%PRNT (titer)**	**WNV 90%PRNT (titer)**	**WNV IFA (titer)**	**DENV 1-4 IFA (titer)**	**YFV IFA (titer)**	**JEV IFA (titer)**
1	**POS**	**POS**	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	230	**1/33**	<1/10	<1/20		<1/20	<1/20	<1/10	<1/10	<1/10	<1/10
2	**POS**	**POS**	**POS**	border	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	>540	**1/164**	**1/1000**	1/20		<1/20	<1/20	<1/10	<1/10	<1/10	<1/10
3	**POS**	**POS**	**POS**	**POS**	NEG	/	/	NEG	NEG	NEG	NEG
	490	**1/243**	**1/3200**	**1/40**				<1/10	<1/10	<1/10	<1/10
4	**POS**	**POS**	**POS**	**POS**	/	NEG	NEG	NEG	NEG	NEG	NEG
	520	**1/127**	**1/3200**	**1/40**		<1/20	<1/20	<1/10	<1/10	<1/10	<1/10
5	**POS**	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	>540	<1/10	<1/10	<1/20		<1/20	<1/20	<1/10	<1/10	<1/10	<1/10
6	**POS**	NEG	/	border	/	/	/	/	/	/	/
	430	<1/10	/	1/20		/	/	/	/	/	/
7	**POS**	NEG	NEG	NEG	/	NEG	NEG	NEG	NEG	NEG	NEG
	265	<1/10	<1/10	<1/20		<1/20	<1/20	<1/10	<1/10	<1/10	<1/10
8	NEG	**POS**	NEG	**POS**	NEG	**POS**	**POS**	NEG	NEG	NEG	NEG
	15	**1/140**	<1/10	**1/80**		**1/320**	**1/390**	<1/10	<1/10	<1/10	<1/10
9	NEG	**POS**	NEG	border	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	35	**1/17**	<1/10	1/20		<1/20	<1/20	<1/10	<1/10	<1/10	<1/10
10	NEG	**POS**	NEG	NEG	/	NEG	NEG	NEG	NEG	NEG	NEG
	15	**1/25**	<1/10	<1/20		<1/20	<1/20	<1/10	<1/10	<1/10	<1/10

Table V-2: Cross-reactivity results for the confirmation panel (TBEV-reactors).

Bold = positive result – not bold = negative or borderline result – NEG: negative result; / : no result due to bad quality or lack of volume sample.

V.4.2 ELISA ACCURACY

a. 2X2 Tables

Two-by-two tables were constructed, depending on RFFIT-SNT thresholds, to interpret the doubtfuls once as true positives and once as true negatives, both times including the ELISA-borderlines as ELISA-positives. The classical accuracy measures were calculated on dichotomized test results (Dohoo et al., 2009b), as summarized in Table V-3.

Summary of 2 by 2 table output for 2 SNT-titer thresholds			
Parameter		**Estimates (95 % exact binomial CI)**	
		SNT cut-off >1/10	**SNT cut-off >1/15**
Test Accuracy ELISA vs. SNT	DSe	0.40 (0.12 - 0.74)	0.57 (0.18 - 0.90)
	DSp	0.91 (0.86 - 0.94)	0.92 (0.88 - 0.95)
	PPV	0.16 (0.05 - 0.36)	0.16 (0.05 - 0.36)
	NPV	0.97 (0.94 - 0.99)	0.99 (0.96 - 1.00)
	LR+	4.34 (1.83 - 10.28)	6.86 (3.20 - 14.68)
	LR-	0.66 (0.40 - 1.10)	0.47 (0.20 - 1.10)
	post-test	0.16	0.18
	Kappa	0.18 (0.07 - 0.29)	0.22 (0.11 - 0.31)
Wild Boar Prevalence	Apparent	0.11 (0.07 - 0.15)	0.10 (0.06 - 0.14)
	True	0.04 (0.02 - 0.08)	0.03 (0.01 - 0.05)

Table V-3: **Summary of 2 by 2 table output for 2 SNT-titer thresholds.**
SNT: RFFIT seroneutralisation test; ELISA: Progen Biotechnik TBE/FSME All species ELISA; CI: confidence interval; DSe/DSp: diagnostic sensitivity/specificity; PPV/NPV: positive/negative predictive value; LR+/-: likelihood ratio positive/negative; post-test: disease probability after a positive ELISA test; kappa: Fleiss' kappa; Sample size: n=238.

The seroprevalence in the whole dataset (n=238) was estimated between 3-4% by SNT and the ELISA had DSe=40-57% and DSp=91-92%, relative to SNT. The positive predictive value was fairly low: we can expect 84% of ELISA-positives to be false in a low-prevalence setting. The negative predictive value is much higher: only a few true positives (1-3%) were missed by this ELISA. The positive likelihood ratio indicates that when wild boar test ELISA-positive, they are 4-7 times more likely to be TBEV-infected (Dohoo et al., 2009b).

Consequently, if pre-test probability of disease is calculated as the percentage of RFFIT-SNT reactor samples (7/238=2.94% - 10/238=4.20%), post-test disease probability for an ELISA-positive becomes 16-18%. Kappa's (0.18-0.22) indicated "poor" agreement with SNT (Fleiss et al., 2003), or "slight" to "fair" agreement (Landis and Koch, 1977).

b. ROC-analysis

ROC-analysis (Figure V-4) showed that ELISA overall discriminatory ability (=AUC) was only 59.74% (95%CI: 32.94%-86.54%) when SNT-borderlines were assumed to be true positives (ST1), and 69.11% (95%CI: 37.21%-100%) when including the same animals as true negatives (ST2). For ST1, the associated DSe/DSp were estimated at 98.68% (95%CI: 96.93-100%) and 40% (95%CI: 10-70%) respectively, and for ST2 the associated DSe=57.14% (95%CI: 14.29-85.71%) and DSp=98.70% (95%CI: 96.97-100%).

The optimal cut-off for this ELISA, determined by Youden's J-statistic (Youden, 1950), turned out to be 155 VIEU/ml. However, with a view to use the ELISA for screening (i.e. with SNT follow-up), the cut-off might be lowered to 35 VIEU: this would result in improved DSe=70-71.43% (for ST1-ST2), at cost of DSp=64.04-69.74% (for ST1-ST2).

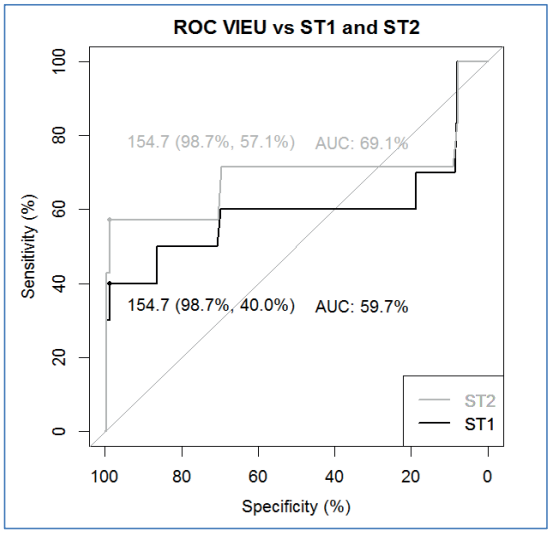

Figure V-4: ROC analysis of the TBEV ELISA as compared to RFFIT-SNT gold standard.
VIEU: standardized Vienna units per ml; animals with SNT-borderline results (n=3) are alternately considered as true positives (ST1: Status 1) or true negatives (ST2: Status 2); Numbers on display represnt ideal VIEU cut-off according to Youden J statistic, (DSp and DSe associated with this cut-off); AUC: Area under the curve: overall discriminatory capability of ELISA.

V.4.3 SEROPREVALENCE AND PROBABILITY OF FREEDOM

SNT-reactor seroprevalence is summarized in Table V-4 and Figure V-5. Seven reactors were localized in Limburg province and three in West Flanders. Borderlines were found in municipalities already containing a seropositive animal. For both provinces (LIM – WFL) and for the whole of Flanders, the Wald and Agresti-Coul confidence intervals excluded 0%, indicating infected populations.

SNT Reactors				
	Sampled	**SNT-Reactors**	**Prevalence**	**95%CI***
WFL	77	3	3.90%	0.88 - 11.30%
LIM	161	7	4.35%	1.20 - 7.50%
FLA	238	10	4.20%	2.56 - 8.31%
*95%CI: if n>100 Wald, if 30<n<100 Agresti-Coul				

Table V-4: **SNT-reactor prevalence in LIM, WFL and Flanders.**
Reactors are SNT -positive or –borderline animals; 95%CI: 95% confidence interval, calculated according to Wald if n>100,or accordingto Agresti-Coul if 30<n<10; WFL: province of West Flanders; LIM: provinces of Limburg and Antwerp – FLA: whole Region of Flanders.

The possibility of freedom despite the observed number of SNT-reactors was further investigated for West Flanders (2 or 3 SNT-positives/reactors), Limburg+Antwerp (5 or 7 SNT-positives/reactors) and Flanders (7 or 10 SNT-positives/reactors), with design prevalences between 0.1-10% and with using a 100% accuracy for the SNT-test. We could not substantiate freedom of TBEV (probability of not infected = 0.000), not even in the conservative case using only the SNT-positives (>1/15). However, the true prevalence can be expected to be below 2.5%.

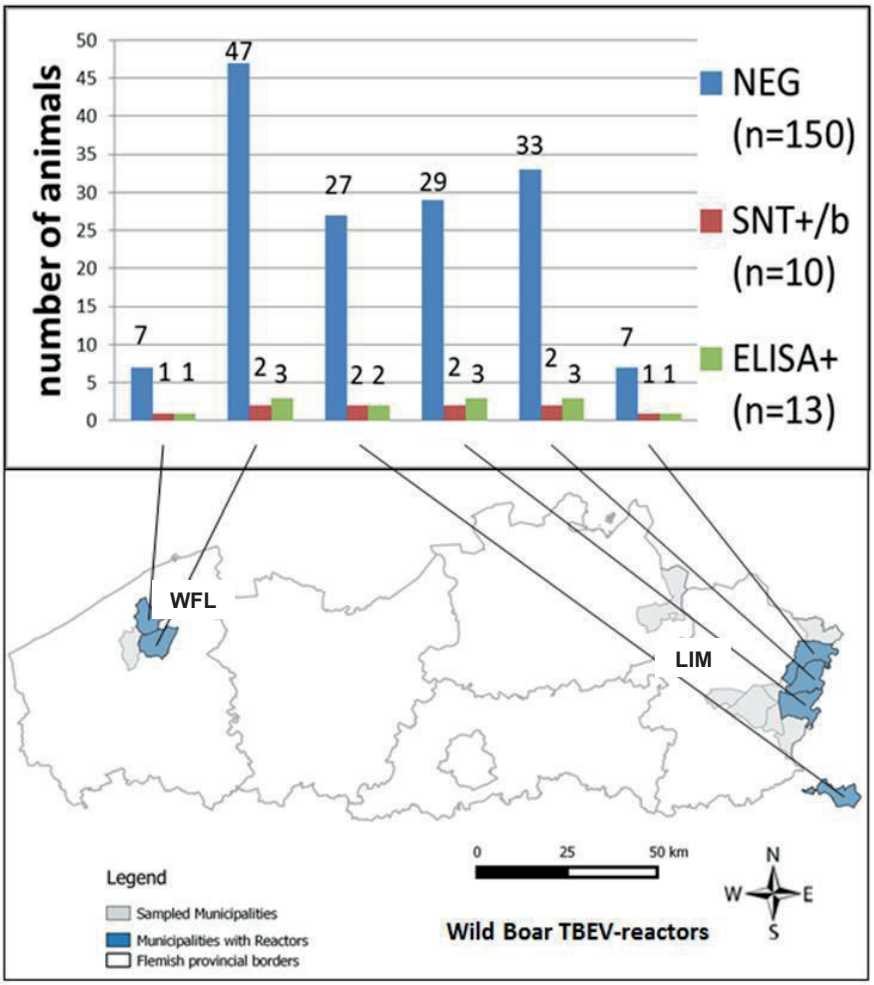

Figure V-5: Reactors in SNT and ELISA tests.

Per sampled municipality (NIS-code); NEG: negative;SNT+/b: seroneutralisation positive (titer>1/15) or borderline (1/10<titer<1/15); ELISA+: enzyme-linked immunosorbent assay positive; WFL: West Flanders subpopulation; LIM: Limburg+Antwerp subpopulation

V.5 DISCUSSION

V.5.1 DIAGNOSTIC TESTING FOR TBEV

Seroneutralisation testing is the gold standard for veterinary TBEV-serology, being highly specific and least affected by cross-reactive antibodies (Klaus et al., 2011; Klimeš et al., 2001; Leschnik et al., 2002; Vene et al., 1998). In this study, ten wild boar reacted positive or borderline in the SNT, three of which presented with very high anti-TBEV SNT-titers (>1/125). This argues for highly specific test results and for classification as true positives. It is nonetheless recommended to check positive SNT-result specificity in comparative tests using a panel of flaviviruses known to circulate in the region (Beck et al., 2013; Klaus et al., 2014).

We selected several flaviviruses to be included in the **cross-reaction tests**. IFA results confirmed the high specificity of the TBEV-SNT: highly SNT-positive sera tested TBEV-positive in IFA, whereas SNT-negative/low positive sera did not. Moreover, ELISA-positive SNT-negative sera did not react in IFA. LIV-HIT revealed several positive reactions, however, the titers were markedly lower than the associated TBEV-titers, and most samples were negative in all other tests. Hence, following worldwide accepted specificity criteria, these reactions were TBEV-specific. Sample nr.8 showed strong cross-reactivity and TBEV-specificity could not be definitively assessed.

None of these **flaviviruses** are considered endemic in Belgium. USUV was found in only two wild birds with neurological signs in southeastern Belgium (Ashraf et al., 2015; Garigliany et al., 2014). Infections or transmission have not been described in suids for DENV/YFV (CFSPH, 2009, 2013; Hubalek et al., 2014), while LIV/TBEV/WNV/USUV may cause asymptomatic infections and were present in Europe before/during 2013 (Ziegler et al., 2015). Extensive Belgian domestic/wild bird WNV-surveillance (serological/virological: n=9,444/3,404) proved negative in the period 2010-15 (FASFC, 2011, 2012, 2013, 2014b, 2015a, b; OIE-WAHID, 2014).

Though few IgM-positives are expected in sentinel populations, Klaus et al. (Klaus et al., 2011) used the Immunozym FSME/TBE All-species IgM-kit, modified by Müller (Müller, 1997) using veterinary Units/L and without IgG-blocking step, to determine total immunoglobuline (IgG+M) in several species, with apparently more accuracy vs. SNT (Klaus et al., 2011).

However, since we experienced insufficient precision or accuracy with this protocol on the bovine sera (unpublished data Chapter IV) and due to low wild boar serum volumes, we opted not to use this protocol for this study.

Using the manufacturer's instructions, the **IgG All-species ELISA** showed low **agreement** with gold standard SNT, with low DSe and reasonable DSp. The threshold could be lowered to increase DSe for screening purpose, but lowering DSp. Ideally, we would have used more positive samples to evaluate the DSe, however, in this low prevalence setting, only n=10 reactors were available. The DSe estimates are therefore only tentative, also indicated by the very wide confidence intervals, while we can be more confident about the actual DSp, based on n=228 negatives. DSe remains to be further evaluated when more positive samples become available, perhaps in an international effort to validate veterinary TBEV-ELISA's.

A first attempt to detect TBEV by **qRT-PCR** in veterinary samples returned negative results. Besides an obvious shortage of available samples (n=13), literature indicates that we should not expect to find TBEV easily. Viraemia lasts only few days in humans (ECDC, 2012; Holzmann, 2003) and mammals (Jaenson et al., 2012; Klaus et al., 2012). Wild boar and pigs are assumed to only develop low-short flaviviral (e.g. WNV) viraemia (Boadella et al., 2012; Chambers and Diamond, 2003; Mandl, 2005; Pripuzova et al., 2013), but in analogy with blood and CSF the virus is probably cleared from lymph nodes by the time humoral response is detectable (Holzmann, 2003; Ruzek et al., 2013).

V.5.2 WILD BOAR AS TBEV-SENTINELS

Though domestic animals are easier to sample than wildlife, the TBEV-seroprevalence in sympatric **wild boar** is in some cases higher, making TBEV-presence easier to detect (Cisak et al., 2012; Gómez-Martínez, 2014). Wild boar feature frequent contacts with rodents and ticks as these species share the same preferential habitats and they provide relatively large serum quantities and cost-effective sampling opportunities (hunting or farming), making them valuable for retrospective surveys and flavivirus/TBEV monitoring (Boadella et al., 2012).

Moreover, for unregulated/growing populations a positive temporal association may exist between human TBE-incidence and numbers of culled boar as this species may be partially driving spread of TBE from woods into suburbs (Kriz et al., 2014).

The 10 TBEV-reactors presented in a male/female ratio of 3/4 (+3 unknown) and their weights (35-98kg) and age ranges (4m-4y) indicated that at least some animals were subadults, capable of dispersal (Dzieciolowski et al., 1990; Markina et al., 2004). We assume that most Flemish wild boar are fairly sedentary, based on average home ranges (≤7km^2), expansion velocity (≤4km/year) and subadult dispersal (≤10km) in Belgium and abroad (Cargnelutti et al., 1992; Gaston et al., 2008; Morelle et al., 2015; Podgórski et al., 2013; Prévot and Licoppe, 2013; Truvé et al., 2004).

However, **subadults** occasionally disperse quite far (≤40km) across highways and national borders (Prévot and Licoppe, 2013; Prévot and Morelle, 2012). Such longer movements may constitute an introduction pathway from TBEV-foci abroad given that adult female *I. ricinus* attach to hosts up to 14 days (Borcic et al., 1990; Kriz et al., 2014). GPS-tracking to define movement patterns of the surveyed population is ongoing (Casaer, pers. comm., 2015). However, given the current information and average Flemish municipality surface (±50km^2), we are confident that in general the Flemish wild boar population is quite suitable to perform local TBEV-surveillance at the community level.

Population size (abundance) is another valuable parameter to assess spread/presence/absence of TBEV within/from foci through epidemiological studies (Prévot and Licoppe, 2013). However, current population estimation methods are biased (Acevedo et al., 2007; Engeman et al., 2013), hence we resorted to expert estimates in our calculations.

V.5.3 Guidance for future Research and Surveillance

To date, no local human TBE-cases have been confirmed in Belgium. However, this emerging vector-borne zoonotic infection has a complicated eco-epidemiology in which human infections only represent the tip of the iceberg (Randolph and Sumilo, 2007). In the light of the observed TBEV-seropositivity in multiple animal species in Belgium, a continued awareness and passive surveillance is indicated to monitor for human cases in general and referral hospitals.

Furthermore, we cannot exclude a degree of underreporting as generally two thirds of human TBE-cases do not feature neurological disease (Süss, 2011). Furthermore, in Belgium general practitioners as well as neurologists are not familiar with TBE (Dr. P. Roelandt, MD, and Dr. M. Goethals, MD Neurologist, pers. comms.).

In such circumstances, veterinary surveillance components add valuable information to existing medical surveillance (ECDC, 2012; Klaus et al., 2011; Klaus et al., 2010a; Süss, 2008b, 2011). For further focus localization and characterization, continued active serological surveillance in several host species is advisable. To attempt virus detection and detection of endemic foci, active surveillance would be recommended in the highlighted areas, by using targeted tick and particularly reservoir sampling and RT-QPCR testing. Indeed, the TBE-virus can be detected for longer periods in several organs of persistently infected wild reservoir rodents (Achazi et al., 2011; Kim et al., 2008; Knap et al., 2012; Pinter et al., 2014) and may be detectable in ticks collected off local hosts, including humans (Bormane et al., 2004; Süss et al., 2006; Süss et al., 2004).

V.6 CONCLUSION

In a cross-sectional serological screening of Flemish wild boar, a positive TBEV-seroprevalence of 2.90-4.20% was found. The seropositive/borderline reactors were clustered in a few municipalities. Three reactors presented with very high and specific anti-TBEV SNT-titers (>1/125) supporting a classification as true positives. Specificity of the SNT was also assessed using a panel of cross-reactivity tests, which ruled out other flaviviral infections for 9 out of 10 TBEV-reactor samples. TBE-virus was not found in qRT-PCR testing of 13 available wild boar tonsils. A commercial TBEV-ELISA was evaluated for its screening accuracy. A fairly low agreement, diagnostic sensitivity and overall accuracy (AUC) were found for the IgG-protocol and the cut-off could be lowered to increase this sensitivity. Diagnostic specificity was found to be adequate For future research, adapted protocols for total immunoglobulin determination may be considered and more positive reference samples need to be collected to further assess DSe.

In conclusion, wild boar can effectively be used for local TBEV-sentinel surveillance in low-prevalence areas. The finding of TBEV-seropositive wild boar in Flanders warrants further follow-up through veterinary and public health surveillance and through targeted direct testing in rodents and/or ticks collected off hosts

CHAPTER VI MAPPING BELGIAN TBEV DATA AND RISK FACTORS

In preparation: Sophie Roelandt, Els Ducheyne, Vanessa Suin, Steven Van Gucht, Stefan Roels, Guy Hendrickx and Yves Van der Stede

QUALITATIVE SPATIAL ASSESSMENT OF HUMAN TBE RISK FACTORS IN BELGIUM

Archives of Public Health (to be submitted in 2016)

BioMed Central - Springer - http://archpublichealth.biomedcentral.com/

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VI.1 INTRODUCTION

VI.1.1 Purpose of Mapping and Modelling TBE

Mapping and predictive modelling are risk-based methods that have been used particularly in vector-borne diseases to identify the areas and time periods in which surveillance is more likely to successfully detect emerging health threats at an early stage (Rodriguez-Prieto et al., 2014). Such techniques greatly benefit governments and public health agencies:

(1) by describing (maps) or analyzing (models) the situation in the field;

(2) by forecasting potential cases and by extrapolation of human risk to areas where no epidemiological data have been collected yet;

(3) by stimulating prevention and mitigation of vector-borne disease, through public campaigns for personal protective actions;

(4) by allowing allocation of limited resources to targeted control, such as vaccination, communication, chemical tick control or habitat modifications (Daniel et al., 1998; Eisen and Eisen, 2011; Kalluri et al., 2007).

Such integrated studies should be performed in interdisciplinary collaboration and are of particular importance especially for those areas where local economies rely on tourism and recreational activities, as the vital "healthy and safe" public image of these areas environment would be damaged by emerging zoonoses (Rizzoli et al., 2009).

VI.1.2 Mapping Examples

Mapping of clinical cases is a very useful tool to spatially allocate TBE surveillance and interventions. It has therefore been used regularly in many countries (Labuda and Randolph, 1999; Rinaldi et al., 2006; Stefanoff et al., 2011; Zeman, 1997). Endemic, risk and high risk areas may be defined based on clinical cases or on seroprevalence and series of risk maps may document the progressive addition of new risk areas, (RKI, 1998, 1999, 2001 2002, 2003, 2004, 2005, 2006, 2007, 2009, 2010, 2011, 2012, 2013, 2014, 2015), including some close to the Belgian borders between 2006 and 2012: see Figure VI-1 (ISW-TBE and Baxter, 2006, 2009, 2011a, b, 2013).

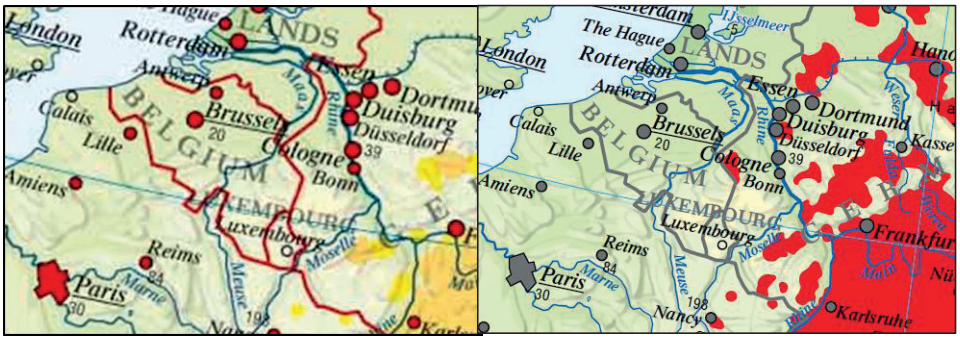


Figure VI-1: TBE/FSME in Europe: Established endemic areas in 2008 vs. 2013.
Adapted from TBE/FSME in Europe: Established risk areas (Baxter/Hölzel Verlag, 2008/2013). Maps based on documented cases of TBE as reported by WHO and national health institutions; Red dots in 2008 and grey dots in 2013: large cities; Yellow areas in 2008 and red areas in 2013: TBEV endemic areas

Case maps should also take into account sentinel animal sampling and seroprevalence and the prevalence in the tick population (ECDC, 2012; Rendi-Wagner, 2004; Süss, 2003): see Figure VI-2 and VI-3.

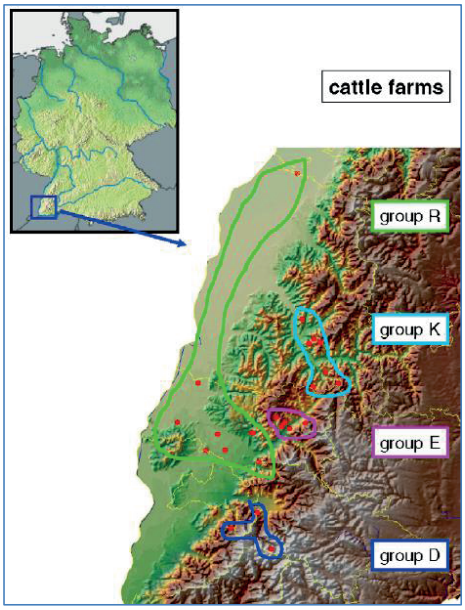


Figure VI-2: Cattle sampling map.
Origin of serum and milk samples (1997-2004) from southwestern Germany; nK=nE=nD=100; n_R=206 (Leutloff et al., 2006)

Figure VI-3: Fox seroprevalence map.
Origin districts of the tested fox sera (first number - light and dark grey); districts with Western-blot positives (number between brackets - dark grey)

Additionally, the important known epidemiological drivers/risk factors of vector-borne diseases can be visualized descriptively and comparatively (Braks et al., 2014). This may include habitat/landscape features and management actions, as well as tick/host distributions and abundance (Li et al., 2012b): Li et al., 2012b): see Figures VI-4 and VI-5 as examples from Sweden and Belgium.

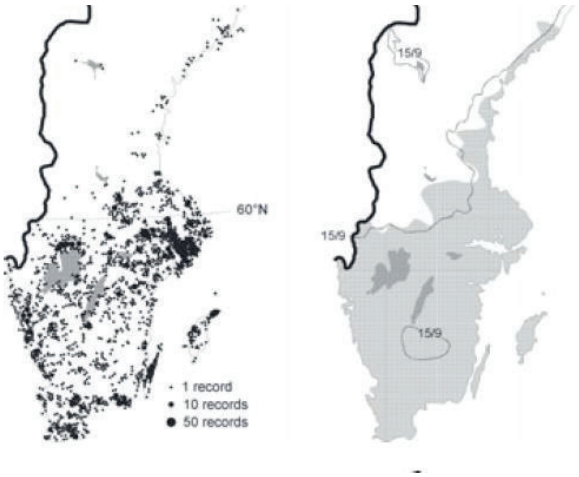


Figure VI-4: Swedish records of Ixodes ricinus ticks (a: left) and estimated distribution versus vegetation zones (b: right).
Dots: tick records 1980s to early 1990s; small dots: individual records; larger dots - 10 and 50 records ; vegetation data (1961–1990): the northern limit of the southern boreal zone (solid line) and the northern limit of the boreo-nemoral zone (dashed line). Adapted from (Jaenson et al., 2009; Jaenson et al., 1994)

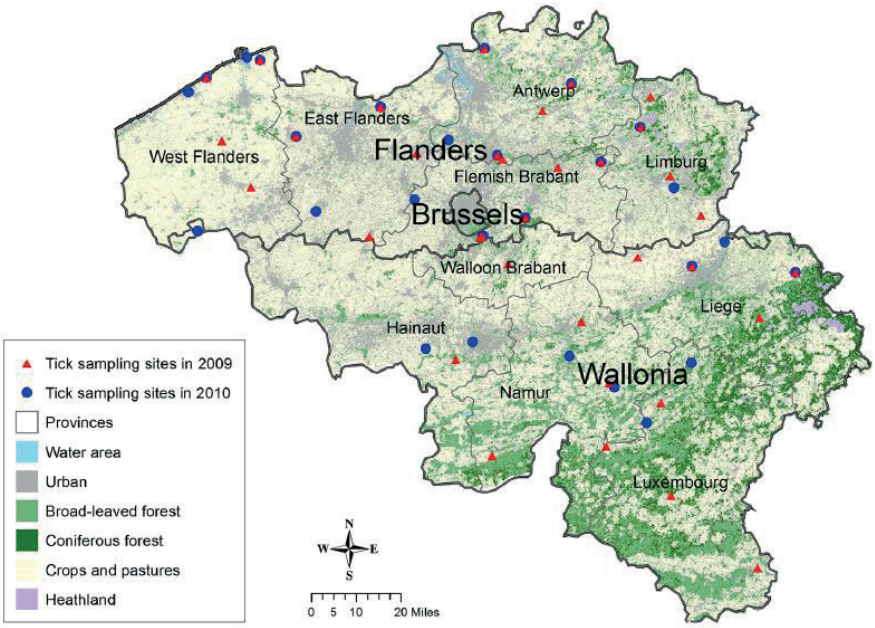


Figure VI-5: Map of Belgium and location of sampling sites in 2009 and 2010.
From (Li et al., 2012b)

VI.1.3 MODELLING EXAMPLES

Predictive models can produce TBE risk maps based on remote sensing data of the important environmental and meterological/climatic drivers, e.g. Figure VI-6 (Randolph, 2000).

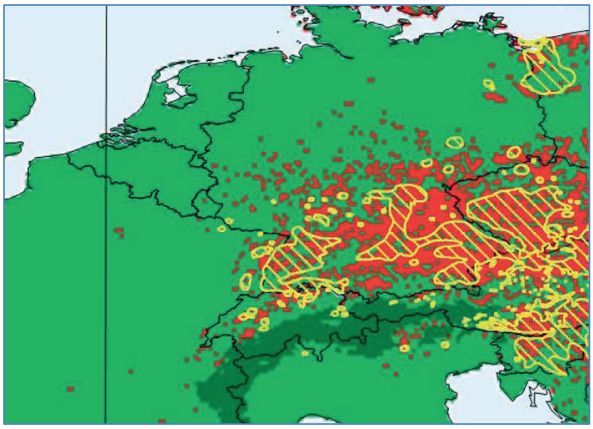


Figure VI-6: Predictive and Spatial Modelling for TBE(V).
Satellite-derived predicted distributionof Tick-borne encephalitis in Western and Central Europe (red, 2000) compared with established foci (yellow, mapped 1997) (From Randolph and Rogers 2000 and Randolph 2001, with permission)

Such predictive modelling assess the risk for vector-borne diseases, by combining different layers of sources related to the drivers/risk factors with field data/observations (recorded cases, tick bites, positive ticks, or seroprevalences). Spatial and predictive modelling are continuously evolving techniques with the goal to reveal the most important risk drivers, and where the explanatory variables (drivers) are often modelled with dependent variables obtained from field data, such as numbers of recorded cases or seroprevalences.

Many different risk factors are known to drive TBE epidemiology (see Table I-1 – Annex VI-4) and in Belgian and European modelling and mapping studies on Ixodid tick abundance, human-tick contacts (tick bites) or rodent-borne zoonotic pathogen prevalence in humans (e.g. Hantavirus, Lyme, TBE), there have been dozens of positively associated and significantly predictive variables (alone or in interactions) and there have been contradictions between the studies. These many correlated variables can be categorized in 7 major groups: meteorological/climatological; landscape structure/cover; landscape configuration; geological/geographical; wildlife; vegetation; socio-economic.

Often the models used are correlational pattern-matching regression models (e.g. negative binomial, linear, poisson, logistic, multilevel, mixed) or discriminant analysis (e.g. principal component analysis, boosted regression trees). Alternatively, mechanistic and machine learning simulation models may also be used (see Figure VI-7) (Eisen and Eisen, 2011; Hartemink et al., 2014; Kalluri et al., 2007; Lambin et al., 2010; Randolph et al., 2000; Randolph and Sumilo, 2007; Reisen, 2010; Zeimes et al., 2014).

Though correlational statistical modelling may suffer from lack of causality and collinearity of the predictors (Hartemink et al., 2014), predictive landscape and meteorological factors are currently easier and cheaper to document spatially for large areas than the many mechanistic causative factors leading to human risk, i.e. abundance of transmission/reservoir hosts (rodents/birds) and the disease burden in ticks feeding on those hosts (James et al., 2013). The mechanistic models require complete quantification and elucidation of the causal relationships in the epidemiological triangle (vector-host-pathogen-environment) and of the (a)biotic factors influencing these (Randolph and Green, 1999; Randolph, 2000; Randolph and Rogers, 2000; Randolph and Sumilo, 2007).

Predictive and spatial modelling has been performed in Belgium, for Lyme disease (Li et al., 2012a; Linard et al., 2007), tick bites (De Keukeleire et al., 2015), Hantavirus (Linard et al., 2007) and *I. ricinus* dynamics (Li et al., 2012b), but not yet for TBEV.

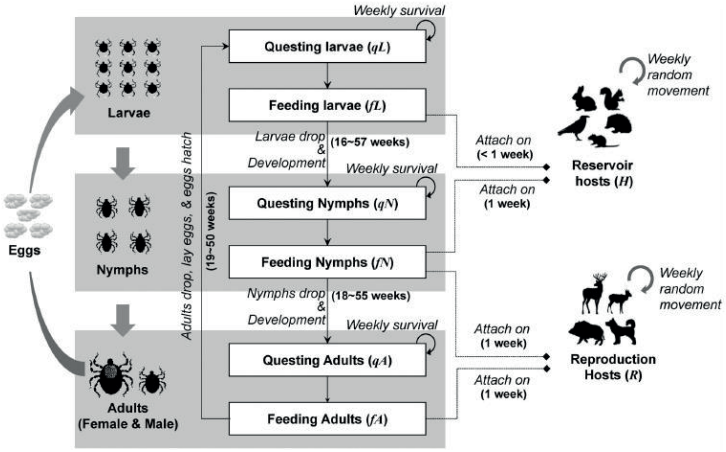


Figure VI-7: Mechanistic model for Lyme disease in *I. ricinus* ticks
solid box: a population stated in the model; solid arrow: development of tick population; dashed lines show attachment relations; white box: questing or feeding stage; larvae feed on small-sized animals, adults feed on large-sized animals, and nymphs feed on both. From (Li et al., 2012a).

VI.2 APPLICATION TO BELGIUM AND TBEV

VI.2.1 SAMPLE AND CASE MAPS

Since Belgium currently has no confirmed autochtonous human TBE cases, we have mapped the sampling (Figure VI-8) and seropositive sample results (Figure VI-9) from the three veterinary sentinel studies conducted as described in chapters III, IV and V (Roelandt et al., 2011; Roelandt et al., 2014; Roelandt et al., 2016)

Besides the seropositive samples obtained in this thesis, 9 human patients with inconclusive results were also added. These cases showed neurological symptoms indicative of neuroborreliosis (Lyme disease) and reacted negative in the neuroborreliosis tests, but positive in the TBEV SNT-test. However, these samples tested negative in TBEV IgM ELISA and could not be conclusively diagnosed as acute TBE cases. Since these medical data were obtained from the referral Lyme laboratory (Cliniques Saint Luc - UCL Brussels – Dr. B. Kabamba, MD), this mainly involves a catchment area around Brussels. Furthermore, the vaccination and mobility/exposure history of these patients is unfortunately unknown (n=9/113; Source data: WIV-ISP; 2009-2013).

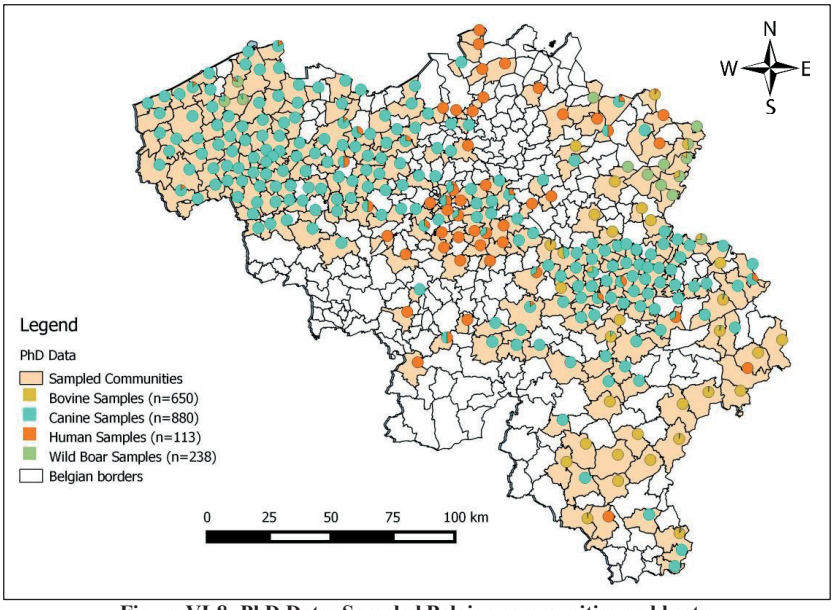


Figure VI-8: PhD Data: Sampled Belgian communities and hosts.
Canine data (Roelandt et al., 2011; Chapter III), Bovine data (Roelandt et al., 2014; Chapter IV), Wild boar data: Roelandt et al., submitted; Chapter VI; Human data (source WIV-ISP; 2009-2013); WGS 84 projection.

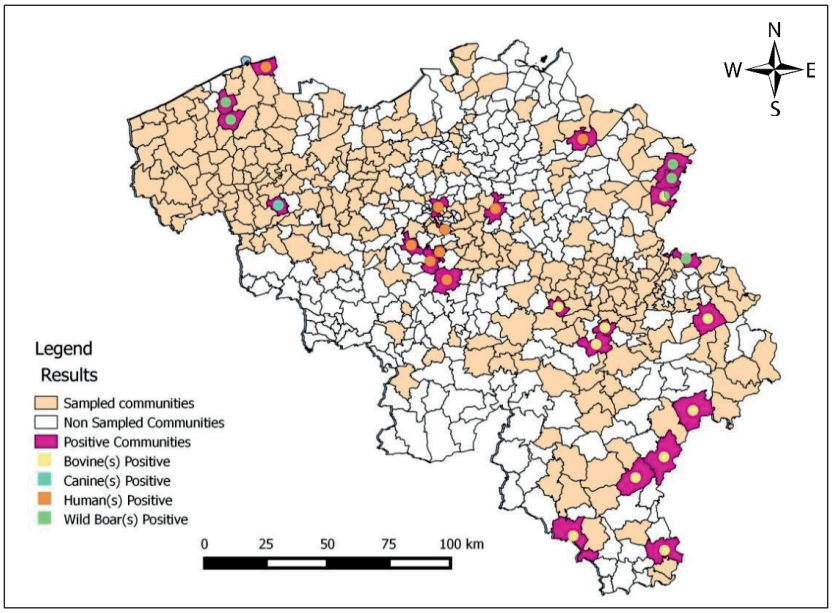


Figure VI-9. PhD Results: TBEV SNT-reactors in sampled Belgian communities.
Canine data (Roelandt et al., 2011; Chapter III), Bovine data (Roelandt et al., 2014; Chapter IV), Wild boar data: Roelandt et al., submitted; Chapter VI; Human data (source WIV-ISP 2009-2013)

VI.2.2 Risk Factor Mapping

In the absence of sufficient data to model TBE risk for Belgium (See under VI.2.3), we decided to proceed by describing and qualitatively evaluating some documented major TBEV-drivers for Belgium. The resulting maps in Figures VI-9-10 were based on the World Borders Dataset (Sandvik, 2009).

From the very abundant literature on TBE risk factors (see Table I-1, Chapter I, and Annex VI.4), eight frequently mentioned risk factors were selected, without any subjectivity on relative importance other than "being mentioned frequently". This means other layers may be used by other studies. Additionally, only those risk factors were selected for which Belgian spatial raster data or predictive model outputs at 1 km^2 pixel resolution was available and easily accessible for the authors during Autumn 2015.

VI.2.2.1 Selected Layers for Risk Factor Mapping

Roe deer and **wild boar** are the most important wildlife hosts for *I. ricinus* and TBEV. For these species, the modelled probability of presence in every 1 km^2 pixel can be used to describe the likely geographical distribution of the species, based on incomplete available data from some pixels. The modeled output of these layers was based on the EMMA (Avia-GIS, 2012), GBIF (GBIF, 2015a, b) and IUCN red list (IUCN, 2012) databases, as well as (sub)national hunting bag data and species-specific GlobCover 2009 (Arino et al., 2012) habitat preferences (Alexander et al., 2015; Alexander et al., 2014).

Cattle and small ruminants are additional important domestic hosts and sentinels. Spatial ruminant distribution data were obtained during the Gloworm FP7 Project (http://www.gloworm.eu/;2012-14) and modeled during the EDENext Project (http://www.edenext.eu/; 2011-14). The final output layer represents the actual abundance per 1 km^2 for each population.

An ***I. ricinus*** tick distribution layer was obtained from the VBORNET project (Wint and Alexander, 2013). Currently, abundance data on **rodents** or other species was not freely available to the authors.The presence or absence of mixed and/or broadleaved forests, which are important to support hosts and vectors, was mapped in 1 km^2 pixels based on the Corine Land Cover raster data 2006, version 13 (CLC, 2006). This type of layer represents **vegetation** and is usually correlated to relative humidity indices, which promote tick stage development.

Distance weighted human population **proximity indices to forests** ("human exposure") were used, reflecting the actual exposure of urban human populations to local forested areas within 30 km Euclidean distance. This layer describes the population which may be likely to visit specific less populated areas, e.g. forests which may receive high urban visitor numbers (Alexander and Wint, 2013; EDENext, 2012).

The **monthly cooling rate during Autumn** (August to December) is an important factor influencing occurrence of synchronised nymphal-larval co-feeding in Spring. temporal Fourier analysis was used (Randolph et al., 2000) on the longterm Belgian monthly maximum temperature data between 1980-2000 (more recent data were not available). These data were used to calculate the Autumn slopes of Land Surface Temperature with temporal Fourier analysis (Randolph et al., 2000; Rogers et al., 1996; WorldClim, 2015).

Fourier analysis is the study of the way general complicated functions may be represented or approximated by sums of simpler trigonometric functions (Wikipedia, 2016). It yields the mean seasonal cycles of the variable of interest, including the amplitude, the phase i.e. timing of peak values, and the shape of the mean seasonal profile (Randolph et al., 2000).

VI.2.2.2 Risk Factor Map Construction

In step one, each of the described spatial layers represents one risk factor for TBE, for which we do not yet know the relative importance (regression coefficients) for Belgium at present. Hence, they were first depicted separately so that one can appreciate factor by factor (Figure VI-11, left).

Since there are no publications on qualitative TBE risk factor maps or on Belgian TBE risk factors, the risk was defined by defining a cut-off using the distribution of most of the different layers. We selected the most extreme 20% quantiles for each layer as the epidemiologically relevant, except for the forests. For the forest layer a binary classifier was used: simple presence/absence of broadleaved or mixed forests.

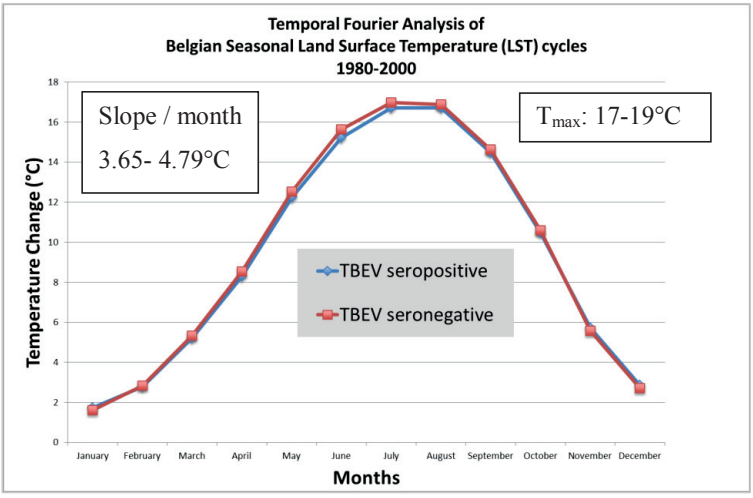
The selection of the most extreme quantiles means the upper (80-100%) quantiles for the host distributions and for the human proximity index as these have a positive association with TBE-risk, or the lower (0-20%) quantiles for Autumn T°slope as here a negative slope (drop in temperature) is associated with increasing TBE-risk. Each selection of 20% represents the more extreme cases at the higher end of the TBE risk scale (risk present~highest). All other quantiles were considered to be not at risk or at very low risk (absent~lowest) (Figure VI-11, right). With this cut-off, we intended to be sensitive enough for each risk factor (include 20%) but also specific enough (not assign TBE risk too easily).

In a third step, the number of risk factors per 1 km^2 was calculated by summing (GIS 'or' operation) the number of risk factors present per pixel. The resulting qualitative TBE risk factor presence map with color code (Range: 0-6) is shown in Figure VI-12, including the serologically positive municipalities, which can be observed in this thesis in a vector layer (red transparant polygon overlay).

Finally, the average number of TBEV risk factors (Range: 0-6: see Figure VI-12) for tested infected municipality was calculated and compared to the average number in the tested free municipalities by the one-sided Mann-Whitney-U test (significant $p<0.05$).

VI.2.2.3 Results

The Belgian monthly maximum temperatures (Tmax: 17-19°C) and negative slopes (between 3.65- 4.79°C per month) in Figure VI-10a are below Prof. Randolph's TBE risk zone in Figure VI-10b: Tmax: >24°C and T° slope: 8-9°C per month (Randolph et al., 2000). Also, there seems to be no difference between Belgian communities with and without TBEV seropositivity.



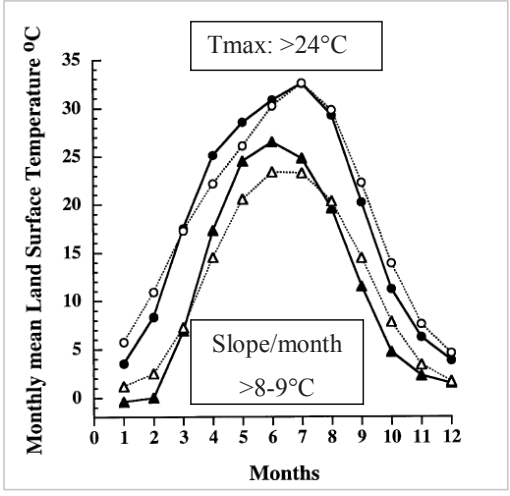


Figure VI-10. Fourier analysis to calculate the Autumn slopes of Land Surface Temperature (LST)
a. Belgian seasonal LST cycles (1980-2000): same slopes for TBEV seropositive and – negative communities 2016; b. Seasonal LST cycles for four European sites (1970's-'80s); black symbols: TBEV present and steaper slopes – white symbols: TBEV absent and gradual slopes (Randolph, 2000)

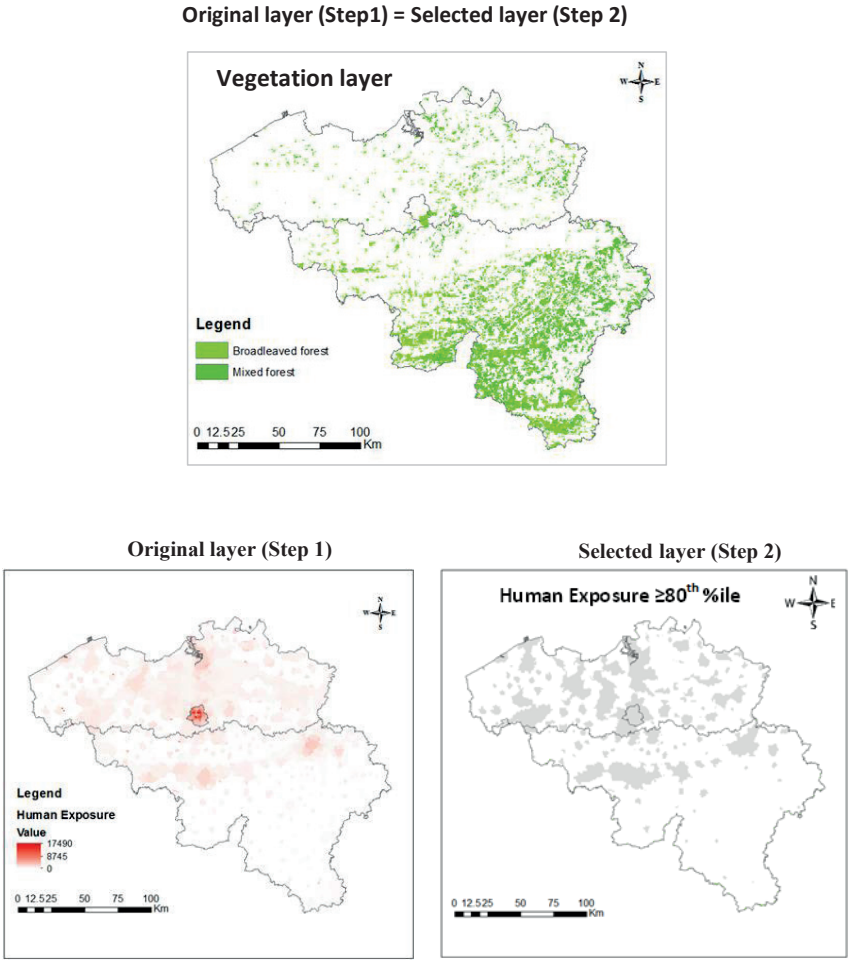


Figure VI-11. Descriptive TBE risk factors / drivers present in Belgium (step 1, left) and at risk pixels (white) versus not at risk pixels (black) per risk factor (step 2, right).

Step1*: Hosts, vectors, landscape and meteorological factors; Full scale between 0-100% probability or min.-max.density/km2.* ***Step 2:*** *Selected pixels at risk (grey) are at or above the 80th percentile (hosts, ticks and landscape factors) or at or below the 20th percentile (negative Autumn T° slope) of the distribution; WGS 84 projection*

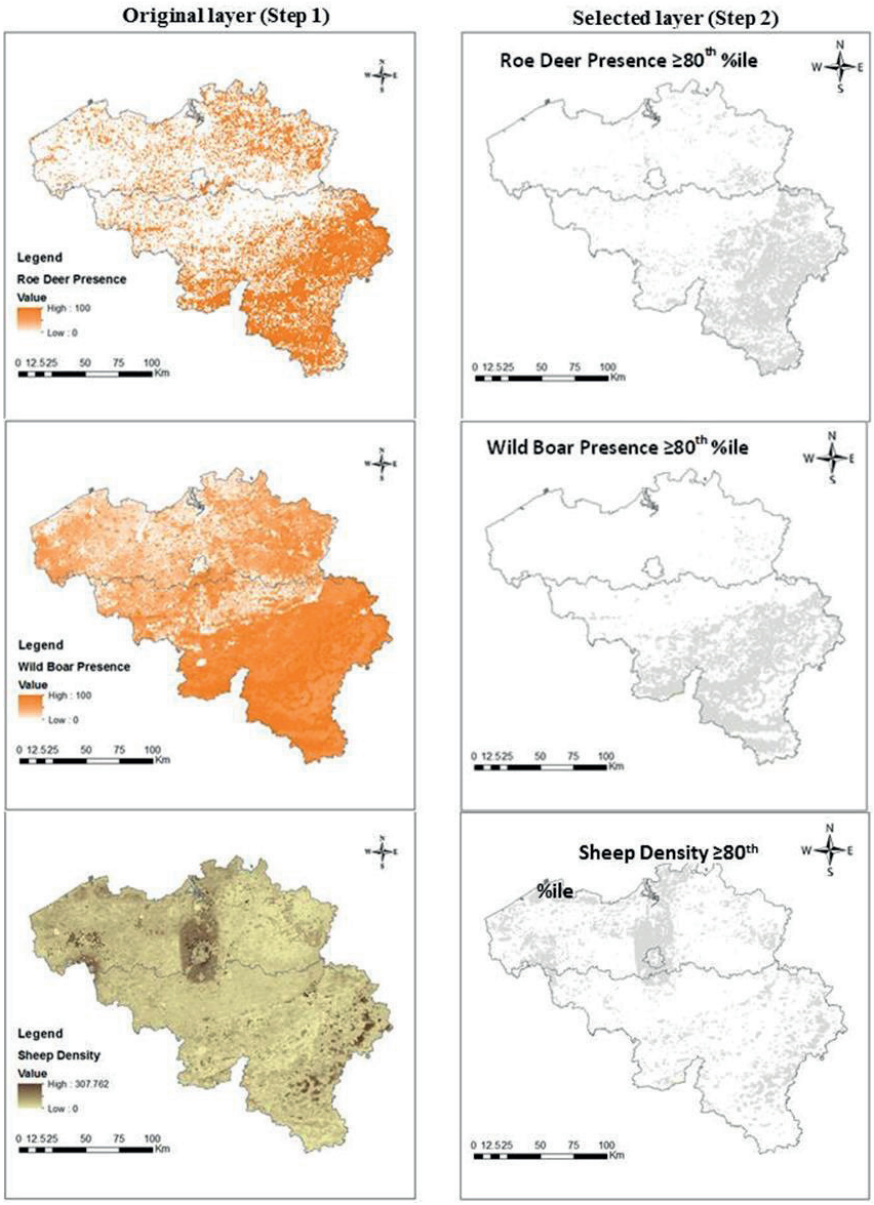


Figure VI-11 (Continued). Descriptive TBE risk factors / drivers present in Belgium (step 1, left) and at risk pixels (white) versus not at risk pixels (black) per risk factor (step 2, right).
Step1: *Hosts, vectors, landscape and meteorological factors; Full scale between 0-100% probability or min.-max.density/km2.* ***Step 2:*** *Selected pixels at risk (grey) are at or above the 80th percentile (hosts, ticks and landscape factors) or at or below the 20th percentile (Autumn T° slope) of the distribution; WGS 84 projection.*

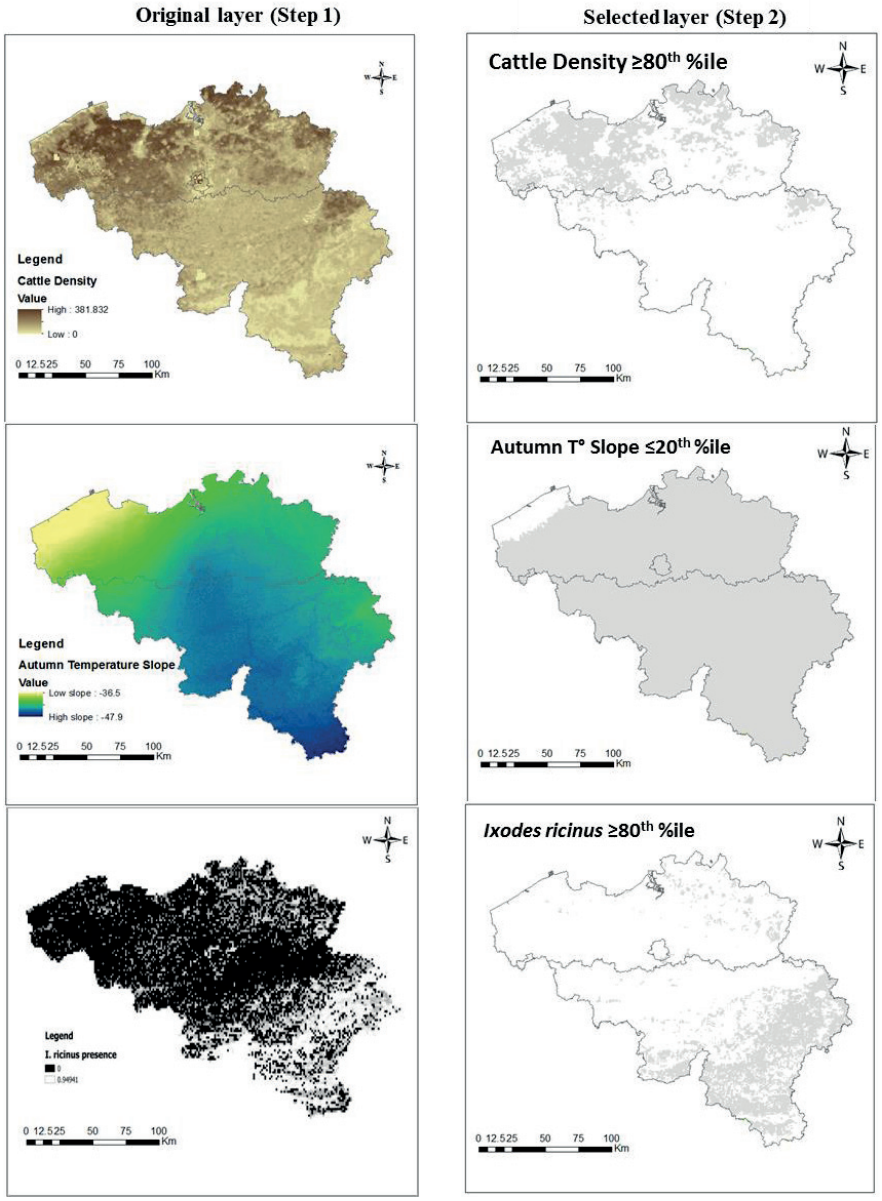


Figure VI-11. (continued) Descriptive TBE risk factors / drivers present in Belgium (step 1, left) and at risk pixels (white) versus not at risk pixels (black) per risk factor (step 2, right).

Step1*: Hosts, vectors, landscape and meteorological factors; Full scale between 0-100% probability or min.-max.density/km2.* ***Step 2:*** *Selected pixels at risk (grey) are at or above the 80th percentile (hosts, ticks and landscape factors) or at or below the 20th percentile (Autumn T° slope) of the distribution; WGS 84 projection*

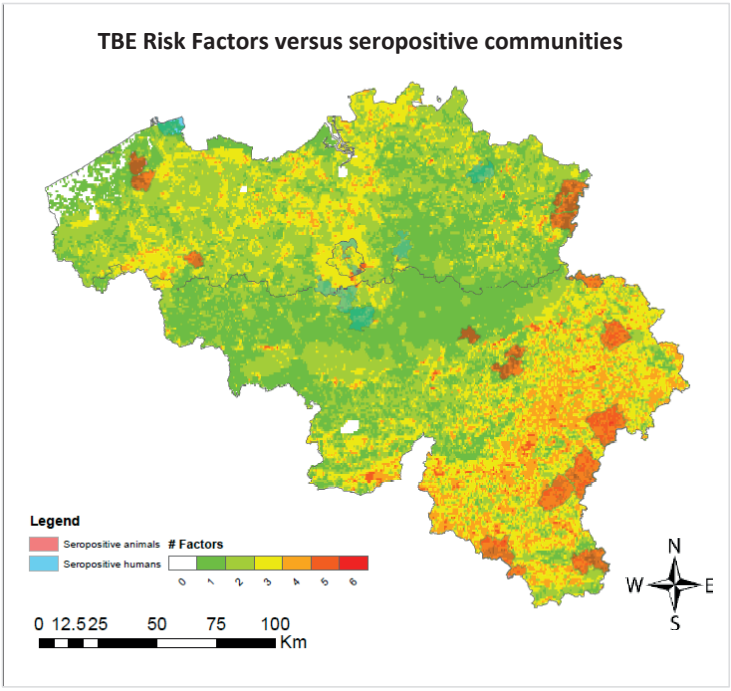


Figure VI-12. Map of TBE risk factors with serolopositive communities (Step 3).
Number of TBE risk factors present in Belgium for 1km^2 pixels (color code 0-6) and Belgian communities with TBEV-seropositive reactions in vector overlay; (veterinary: red polygons; medical: blue polygons).

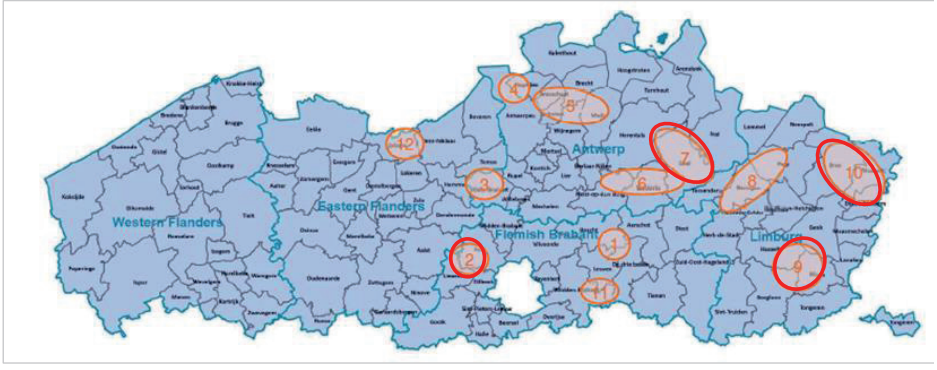


Figure VI-13. Map of TBE positive roe deer in Flanders, adapted from Tavernier et al. (2015).
Orange circles: these zones were sampled (n=11); Red circles: the TBEV-seropositive zones were no. 2 (n=1/4) , no.7 (2/24) , no.9 (1/11) and no.10 (1/47).

The cattle cases in southern Belgium do overlap with the roe deer cases in Liège and Luxembourg (data not shown; Dr. Linden A., pers.comm.). Comparing the Figures VI-9, VI-12 and VI-13, there are also matching case patterns *i.e.* overlaps in Limburg for cattle (Roelandt et al., 2014), roe deer (areas no. 9-10) (Tavernier et al., 2015) and wild boar (Roelandt et al., 2016). There is a further overlap between roe deer (area no.7) and humans in Antwerp province. These overlaps are some further substantiation of true TBEV endemic foci in these areas.

The human inconclusive seropositives around Brussels may indicate a potential relationship with the Sonian forest that contains well-known recreational areas full of suitable TBEV hosts, vectors and habitat. So far this has not been matched with positive dogs (Roelandt et al., 2011), but one roe deer was TBEV-seropositive in Flemish Brabant (Tavernier et al., 2015); the other species were not yet sampled in this area. Furthermore, the medical data need to be interpreted with great care, since the suspected patients mainly came from the same hospital (Cliniques St. Luc, ULC, brussels): there is some selection bias. And since the patients' flaviral vaccination histories were unknown, these results may still indicate a population vaccinated against e.g. TBEV or YFV and traveling internationally.

Statistically, the p-value for the one-sided Mann-Whitney-U test for all species together (NIS_{pos}=26 vs NIS_{neg}=268) was not significant (p=0.9342). The one sided p-value for cattle + wild boar (NIS_{pos}=15 versus NIS_{neg}=30) was statistically significant ($p<0.01$), indicating that veterinary (cattle+boar) seropositive communities are associated with a higher number of TBE risk factors, but not so for dog and human seropositives.

VI.3 DISCUSSION

VI.3.1 Risk Factor Mapping Methods

We considered many potential data layers, but many were not available at short notice and in a raster format. Despite the plethora of potential risk factor variables to choose from (Table I-1 and Annex VI.4), the current selection covers the three main categories (**bold**) of TBE risk factors (Lambin et al., 2010):

(1) Life cycles and population dynamics of pathogens, **hosts and vectors**;

(2) Landscape structure and **composition**, configuration and connectivity;

(3) Human **behaviour** and land use.

For this first qualitative TBE risk mapping at the scale of 1 km^2 Belgian pixels, the counting of quite broadly defined "presence/absence" risk factors was used, as opposed to fully quantitative modelling of the continuous raster data (Belgium = 30,528 x 1km^2 pixels). Quantiles can be applied to such continuous distributions, providing a way to allow rank statistics of these variables, such as Mann-Whitney-U test (syn. Wilcoxon rank-sum test).

In our approach, we arbitrarily defined the risk factor's presence or absence (ranks) based on the more extreme quantiles of the observed continuous distributions (20% upper or lower values). This might not be totally realistic, but with the real Belgian risk factor regression coefficients missing and with insufficient case data, this dichotomization per risk factor was the only approach feasible at this time; the 20% cut-off is meant to be sufficiently sensitive/specific.

The dichotomous cut-offs and the summing of risk factors with the same weigth/coefficient (=1) are in fact a simplification of logistic regression treatment of predictive variables, to obtain ordinal colour ranks according to the number of risk factors present. Together with overlay of the seropositive communities (available case data in vector format) was the only mapping/regression approach feasible with the available data at this time.

The original cattle and wild boar studies were risk-based at the province selection level, based on the risk of TBEV introduction from Germany. However, at the community level, all the available serum samples were used, independent of animal densities or other TBEV risk factors, allowing for random selection at this level and comparison of risk factors at the community level within those selected regions. However, as was concluded after each of the serology studies, the local/regional TBEV seroprevalence should not be extrapolated to the whole Belgian territory without further surveillance and verification.

Despite these limitations, the observed seropositive Belgian communities in all species do seem to fall within the arbitrarily defined "at risk" zones that are associated with a qualitatively higher number of risk factors (orange-red zones: 4-6 risk factors). For the cattle and wild boar data, there was a statistically significant association in the MWU-test.

Additionally, we also included human, roe deer, sheep risk, vegetation and meteorological factors. The output thus allows us to hypothesize that cattle and wild boar exposure is to be expected where more of these other factors are present. Provided that a much larger number of confirmed Belgian cases/infections can be obtained for the whole territory, these risk factors could be confirmed or refuted, as has been done in other countries.

Predictive and spatial modelling would provide a much more detailed and precise insight (real regression coefficients) on the spatial distribution of Belgian TBE risk, and of the relative importance of the different risk factors, much as done in other Europan TBEV, Lyme and tick modelling studies (see VI.1.3. and Annex VI.4).

VI.3.2 Prediction Versus Reality

In Figure VI-6 and in the accompanying models of Randolph et al. (Randolph, 2000; Randolph et al., 2000; Randolph and Rogers, 2000), using remote sensing data of well known TBEV climatic risk factors, it was predicted that TBEV would not spread so far westwards. Belgium should never contain TBEV endemic areas, although the scenario was not completely excluded. The biological/epidemiological basis for this prediction was the predicted absence of co-feeding of nymphs and larvae due to some climatic requirements (temperature slopes) (Prof. Dr. Randolph, pers. comm., 2009).

Currently, sixteen years after these models and predictions, five Belgian sentinel serology studies point towards TBEV presence in Belgium, against all expectations. A "co-presence" of overwintered nymphs with new larvae has been observed in Belgium during late Spring (May-June) in diverse vegetation types (Tack et al., 2012). It would still need to be confirmed whether these nymphs and larvae actually "co-feed" on hosts during this period.

Our calculations on The Belgian monthly maximum temperatures (T_{max}: 17-19°C; data not shown) and negative Autumn slopes (between 3.65-4.79°C per month) are indeed objectively below the previously defined risk zone for co-feeding: T_{max}: >24°C – Autumn slope: 8-9°C per month (Randolph et al., 2000). This would seem to exclude TBE zones in Belgium, however, our temperature data were based on the years 1980-2000.

In the mean time, climate change (hotter/longer summers, warmer winters, increased annual rainfalls) may indirectly have played a role in the increase of the number of Belgian Lyme disease cases ($n_{1991} = 137 \rightarrow n_{2000} = 1{,}442$) by favouring vector development (Clement and Van Ranst, 2002 ; Hoyaux et al., 2011) and could potentially lead to introduction of diseases such as TBE into new places (BELSPO-VGT10; Hoyaux et al., 2011).

Since we defined risk as the 20% most extreme of the Belgian temperature slope ranges, a large part of Belgium remained in the defined risk zone for this study. Perhaps the temperature dichotomization might have been a little more strict (5-10% most extremes as risk zone), to exclude more of the Belgian territory, although there is no objective reason for or against this, until the potential for co-feeding and more recent temperature (slope) data are re-assessed for Belgium.

Additionally, very recently (June 2016), a Dutch-Belgian research team has detected the first ELISA- and SNT-positive Dutch roe deer in sera collected during 2010. Importantly, TBE viral RNA was also detected in two questing ticks from the same location in 2015 (Jahfari et al., [submitted]), demonstrating the presence of TBEV in the Low Countries (60km from the Dutch-German border), where it was not predicted.

VI.4 ANNEX RISK FACTORS*

*Significant risk factors found in EU and USA mapping
and predictive modelling studies on TBE – Lyme –Ticks – Reservoir/Host

VI.4.1 METEOROLOGICAL AND CLIMATOLOGICAL

High daily maximum temperature, low daily wind speed, low daily relative humidity and high daily vapour pressure deficit (BE) (Li et al., 2012a); high number of days with max. temp 24°C, high precipitation in the last two months (BE)(De Keukeleire et al., 2015); land surface temperature (EU; Randolph, 2000); low humidity/precipitation (UK) (James et al., 2013); mean summer temperature above 12°C (SW) (Palo, 2014); autumnal cooling rate (IT)(Rizzoli et al., 2007); amount of precipitation (snow) in December (SW)(Haemig et al., 2011); temperate climate with high relative humidity and seasons (USA) (Falco et al., 1999); increase in winter temperatures rainfall (USA) (Estrada-Pena, 2002b); 8°C annual isotherm and mean rainfall 800mm (EU)(Labuda et al., 1997a; Labuda et al., 1997b; Labuda and Randolph, 1999); maximum, minimum, and mean temperatures and vapor pressure (USA) (Brownstein et al., 2003); no effect fast spring warming (D)(Kiffner et al., 2010); effect fast spring warming (EU) (Randolph and Sumilo, 2007); mild winter and high summer temperatures, and low seasonal amplitude of temperatures (EU) (EEA, 2012); higher temperature promotes tick + higher humidity promotes virus replication in ticks (EU)(Sonenshine and Mather, 1994); dry and hot spring may disrupt cofeeding (CH)(Burri et al., 2011); February to May the temperature rises rapidly >10°C (larvae) and >7°C (nymps) at 60cm above soil (Randolph, 2004) and simultaneous dry weather(Randolph and Storey, 1999); mild winter followed by hot/dry spring decreases nymphs (CH)(Burri et al., 2011); higher rate of autumnal cooling (mean monthly LST august – October) (Randolph, 2001); milder winters less days of < -7°C and extended spring/autumn with >5-8°C (SW)(Lindgren and Gustafson, 2001; Randolph and Rogers, 2000); >85% relative humidity = broadleaved litter layer; (Randolph, 2000);

NDVI annual amplitude, amplitude/phase of LST, middle infrared radiation , ground temperature (Randolph, 2000).

VI.4.2 Landscape Structure

High proportion of broadleaved forest (BE) (Li et al., 2012a); high proportions of forest and broadleaved forest, agriculture, artificial/urban, peri-urban; low proportion of arable land or water and high proportion of artificial land of semi-natural habitats bordering forest (BE) (De Keukeleire et al., 2015); high proportion of forest, low proportion of agricultural land, smaller villages with smaller less aggregated human populations (HU)(Racz et al., 2006); high proportion of forests and high proportion spatially dispersed larger houses (BE) (Linard et al., 2007); environment/land cover is suitable for tick and tick-host populations (LT; Wanwambeke et al., 2010); highly fragmented woodlands, low proportion of adjacent grasslands (BE; Li et al., 2012b); diverse broadleaved or mixed forest with water bodies, holiday houses and open areas/clear-cuts (SW: Zeimes et al., 2014); green vegetation or proportion of woods, less developed/urbanised region (USA) (Glass et al., 1995); more greenness (relative measures of vegetation structure and moisture) and wetness (vegetation abundance) (USA) (Dister et al., 1997); high risk in mixed and deciduous woods with shrubs versus low risk in coniferous woods (CZ) (Daniel et al., 1998); land cover (USA) (Bunnell et al., 2003); proportions of broad-leafed, mixed and coniferous forest cover (D) (Kiffner et al., 2010); protective effects agricultural land, low residential housing, flat land, inside of forest versus risk at forest edge (USA) (Das et al., 2002); higher vegetation indices in the May-June period (EU) (EEA, 2012); broadleaved forest >> coniferous/non-wooded (Randolph, 2000).

VI.4.3 Landscape configuration

High forest edge density, high area-weighted mean forest shape index, high forest patch fractal dimension, low Euclidean mean nearest-neighbour distance between forest patches (BE)(Li et al., 2012a); high proportion edge forest-artificial land, low proportion edge forest-water, high edge density, low distance to nearest forest patch (BE) (De Keukeleire et al., 2015); mixed landscapes in peri-urban areas (Lyme) versus remote forest areas with low urbanization (hanta) (BE)(Linard et al., 2007); density, shape and aggregation level of woodland patches (BE) (Li et al., 2012b); well-connected forest with complex shape (SW) (Zeimes et al., 2014); residential properties close to wooded areas (USA) (Glass et al., 1995); deciduous forests with leaf litter or grass lands with shrubs and tall grasses (USA) (Falco et al., 1999); increase in woodland in close proximity of human habitation (USA) (Wilson et al., 1985); more in deciduous, dry to mesic forests and less in grasslands, conifer forests, wet to wet/mesic forests (USA) (Guerra et al., 2002); heterogeneity of inner wood structure (CZ)(Daniel et al., 1998); distance to water, distance to forest edge (USA) (Bunnell et al., 2003); larger and less isolated forest patches have higher human risk but smaller more isolated patches have more ticks and tick infection prevalence (USA)(Brownstein et al., 2005); forest fragmentation to small patches (D) (Kiffner et al., 2010); and well-connected vegetation patches (EU) (EEA, 2012); fragmented forest, patchiness, <1-2ha (USA)(Allan et al., 2003); areas with high forest, high NDVI, and moisture next to housing, agriculture, recreation (Randolph, 2000).

VI.4.4 GEOLOGICAL - GEOGRAPHICAL

Proportion of clay soil (as opposed to gravel, sand, silt) (BE) (Li et al., 2012a); low altitude (UK) (James et al., 2013); more on alfisol-type soils of sandy or loam-sand textures overlying sedimentary rock and less on acidic soils of low fertility, a clay soil texture and Precambrian bedrock (USA) (Guerra et al., 2002); elevation and soil type (USA) (Bunnell et al., 2003); increased risk on slopes (USA)(Das et al., 2002).

VI.4.5 WILDLIFE POPULATIONS

Roe deer hunting bag (BE) (Li et al., 2012a); changes in wildlife management practices leading to increase in roe deer (IT)(Rizzoli et al., 2009); roe and red deer abundance (UK) (James et al., 2013); wild boar population density much better than roe deer density (CZ) (Kriz et al., 2014); roe deer abundance/density (BE) (Linard et al., 2007); local host spatial patterns and movement (BE) (Li et al., 2012b); female > male in moose/roe deer/wild boar ; male > female in fallow deer ; older age ; areas with low/absent roe deer population may not be free (SW) (Gómez-Martínez, 2014); abundance of European hare and red fox best predictors (SW) (Palo, 2014); wildlife abundance of unstable populations (Knap and Avsic-Zupanc, 2013); high abundance of fox and 1ow abundance of mink (SW) Haemig (Haemig et al., 2011); high abundance of wild boar, red/fallow deer versus low abundance of roe deer (SW)(Zeimes et al., 2014); increase in deer (USA: white-tailed deer) (Wilson et al., 1985); Increase in rodents (USA: white-footed mouse) (Allan et al., 2003); roe deer density and abundance of large adult animals (D)(Kiffner et al., 2012); positive effect hunting bag roe deer, no effect hunting bag red deer, negative effect hunting bag red fox, all in the previous year (D) (Kiffner et al., 2010); increases in roe deer (EU) (Randolph, 2001).

VI.4.6 VEGETATION STRUCTURE

Structure-rich oak/deciduous forests, shrub cover, number of deer beds (BE) (Tack et al., 2012); normalized difference vegetation index NDVI (EU) (Randolph, 2000); vegetation structure + suitability for small mammals (IT)(Rizzoli et al., 2009); deciduous woodland type, high ericaceous/grass/herb/moss ground vegetation (UK) (James et al., 2013); oak, birch and pine forests with tree hight variation of ≥5m (SW) (Zeimes et al., 2014); NDVI ~ vegetation vitality (Estrada-Pena, 2002b); moist mat of undergrowth, broadleaved forests (Randolph, 2001).

VI.4.7 SOCIO-ECONOMIC

High income, separated large houses (Lyme), low income (Hantavirus-rodents) (BE) (Linard et al., 2007), low income ⇔ recreation (TBE); population is likely to enter the forest on a regular base, accessibility and land ownership (Latvia)(Vanwambeke et al., 2010)); accessibility and land ownership (BE) (De Keukeleire et al., 2015); accessibility: roads, holiday houses, attractiveness of forest (SW)(Zeimes et al., 2014); decrease in hunting (USA); (Wilson et al., 1985); awareness (Randolph, 2001).

CHAPTER VII GENERAL DISCUSSION

In preparation: Sophie Roelandt, Vanessa Suin, Steven Van Gucht,

Yves Van der Stede, and Stefan Roels

COMPARATIVE TBEV SURVEILLANCE IN BELGIUM DURING 2009-2015:

EXPERIENCES REGARDING DIAGNOSTIC TESTS,

VETERINARY SENTINEL SPECIES AND SURVEY DESIGNS

Review Paper requested by Veterinary Sciences (to be submitted in 2016)

MDPI Publishers - http://www.mdpi.com/journal/vetsci

VII.1 AIMS AND FINDINGS OF THE THESIS

VII.1.1 Evidence For TBEV Presence In Belgium (PhD Aims 1-2)

Serology is internationally accepted as sufficient evidence to diagnose clinical cases, and for contributing complementary or sufficient evidence to document endemic areas, according to the internationally accepted definitions of "TBE case" and "endemic area" (Amato-Gauci and Zeller, 2012; ECDC, 2012; Süss, 2011; Süss et al., 2010).

In this thesis the evidence "pro" TBEV-presence is indirect: it was obtained through three veterinary serological studies (Chapters III, IV, V; Table VII-1). Due to the encountered problems such as cross-reactions and specific validation problems for implementing ELISA protocols for TBEV, a seroneutralisation test was used in all studies for detection and confirmation of TBEV-specific antibodies.

This SNT test is internationally accepted both in the medical and veterinary communities as the current gold standard for TBE diagnosis and surveillance purposes. Furthermore, in Belgium the SNT is performed under specific accreditation and it has scored well in international proficiency tests (PT report, pers.comm, V. Suin, WIV-ISP). Although no test or laboratory is ever 100% perfect, discarding all positive samples from this thesis as false positives would mean that the gold standard is only 98.26% specific. In this case, the observed seropositives would all be falsely positive: 1 positive dog (1/880), 17-23 positive cattle (17-23/650), 7-10 wild boar (7-10/238) and 7 deer (7/596).

All the efforts undertaken in this thesis (Chapters III, IV and V) to disprove the positive SNT results i.e. to exclude false positive reactions represented important additional work. This consisted of batteries of cross-reaction tests for all studies and an animal infection experiment in the cattle study. Indeed, in many other studies positive ELISA results are often considered unquestionable proof of TBEV-endemicity/positivity (See Tables I-3 and I-4).

In each serological survey conducted in this thesis, it was found that most TBEV-seropositive animals were not clearly positive for any other pathogen. Additionally, several animals had very high antibody titers (>1/125) and the bovine antibodies were effectively neutralising and protecting mice against virulent TBEV in an *in vivo* challenge.

Aspecific reactions are very rare in SNT, and since we have no reason to assume travel/dispersal-related infections in the cattle and wild boar, we may conclude that at least some of these animals were really exposed to TBEV in Belgium or very close to its borders.

Based on Chapters III, IV and V (Roelandt et al., 2011; Roelandt et al., 2014; Roelandt et al., 2016) and the additional knowledge from two other Belgian sentinel studies (Linden et al., 2012; Tavernier et al., 2015), TBEV has been present in Belgium from at least 2010 onwards. The current absence of a characterized TBEV-strain and of confirmed human/veterinary clinical cases is likely due to a lack of surveillance effort and sensitivity, and a lack of additional investigation or follow-up in focal areas where TBEV-specific antibodies were detected.

Available evidence for TBEV presence in Belgium, anno 2016.					
Species	***Seroprevalence***			***Sample***	***Reference***
	SNT+	***SNT +/-***	***ELISA+***		
Dogs	0.11%	0.00%	1.13%	n=880	**Roelandt et al., 2011**
Cattle	2.61%	0.92%	3.85%	n=650	**Roelandt et al., 2014**
Wild Boar	2.91%	1.26%	5.46%	n=238	**Roelandt et al., 2016**
Roe Deer	0.4%	0.00%	12.4%	n=498	Linden et al., 2012
	5.1%	0.00%	/	n=98	Tavernier et al., 2015

Table VII-1: Available TBE Sentinel Data in Belgium anno 2015.
SNT: seroneutralisation test; (+: positive result; +/- doubtful);
ELISA: enzyme-linked immunosorbent assay; n: sample size

In addition, the risk map as produced in Chapter VI, has demonstrated in a qualitative way that the overlayed seropositives match with a higher number of risk factors. For the cattle and wild boar data, there was a statistically significant association. Additionally, there exist geographical **overlaps** between areas with seropositive Flemish cattle, roe deer and wild boar (Figures VI-9, VI-12 and VI-13), and between Wallonian cattle and roe deer (data not shown).

Furthermore, the very recently discovered ELISA/SNT-seropositive deer in the Netherlands were also sampled in 2010 and the Dutch TBEV-positive ticks from 2015 were found at ~60 km of the Dutch-German border (Jahfari et al., [submitted]), hence also in an area in a country very similar to Belgium where emergence was not really foreseen (Gould et al., 2004; Randolph and Green, 1999; Randolph, 2000; Randolph et al., 2000; Randolph et al., 1999; Randolph and Rogers, 2000).

All these results suggest that the 16 year old paradigm of co-feeding - where co-feeding transmission alone determines the persistence or absence of TBEV endemic foci - may not be applicable in the Low Countries and should be re-investigated. Alternatively, perhaps co-feeding currently does occur in this evolving climatical and ecological setting, as nymphs and larvae have been co-present in Spring (Tack et al., 2012). This geographical region of Europe at the edge of the TBEV distribution has a more coastal climate than the TBE core zones in Central Europe, but climatic and other parameters still fall within TBEV's and *I. ricinus'* broad requirements (Hoyaux et al., 2011; Roelandt et al., 2010; Tack et al., 2012).

VII.1.2 Evaluation of Veterinary Tests (PhD Aim 3)

VII.1.2.1 TBEV-ELISA for Screening

The **IgG protocol** of the commercial ELISA Immunozym FSME IgG All Species (Progen, Biotechnik, Heidelberg, Germany) has been used by most veterinary researchers in several species (see Tables I-3-4): dogs, horses, goats, foxes, wild boar, cattle, ruminant milk, deer, monkeys, rodents and moose.

In the studies presented **in this thesis** (Chapters III, IV, V), the same Progen ELISA kit was used to detect TBEV-specific IgG antibodies in the collected animal sera. This non-competitive indirect assay uses horseradish peroxidase – Protein G conjugate to detect any IgG against whole TBE-virus and can theoretically be used for TBEV testing in all species. In humans, this ELISA was shown to have a diagnostic sensitivity of 97% and analytical specificity of 99% for IgG (Progen, 2006).

Despite the claim of being an "All-Species" ELISA, and despite the IgG-protocol being often used internationally, we experienced problems in obtaining accurate test results from all three selected species with the IgG kit, when compared to the gold standard serological TBE seroneutralisation test (Chapters III, IV, V; Table VII-2). We obtained less than satisfactory results, certainly for cattle, where even the measures of precision were suboptimal (repeatability/reproducibility coefficients of variation >20).

Since in validation studies the DSe is usually determined in a larger positive population, i.e. with higher prevalence, our DSe may be overly pessimistic due to the small number of positives obtained in the studies. However, it is clear that the Immunozym FSME IgG All Species kit will miss true positives in a population where the prevalence is moderate to low. As such, the IgG protocol may need considerable adjustments before being suitable as a sufficiently sensitive first-line and large-scale screening test.

Diagnostic Test Accuracy of the All-species IgG-ELISA.				
Species	***Parameter***	***Results***	***Sample Size***	***Reference***
Dog	DSe	1.00 (1/1 pos)	n=1	Roelandt et al. (2011) n=880; **Chapter III** **Negatives not confirmed**
	DSp	0.98 (9 false positives)	n=879	
Cattle	DSe	Min: 0.13 (0.00 - 0.27) Max: 0.17 (0.00 - 0.21)	n=18-23	Roelandt et al. (2014 - unpublished) n=650; **Chapter IV**
	DSp	Min: 0.89 (0.87 - 0.92) Max: 0.97 (0.96-0.98)	n=627	
Wild Boar	DSe	Min: 0.40 (0.12 - 0.74) Max: 0.57 (0.18 - 0.90)	n=7-10	Roelandt et al. (2015) n=238; **Chapter V**
	DSp	Min: 0.91 (0.86 - 0.94) Max: 0.92 (0.88 - 0.95)	n=228	

Table VII-2: Diagnostic Test Accuracy of the All-species IgG-ELISA.
As compared to TBE-SNT as gold standard. DSe/DSp: Diagnostic Sensitivity or Specificity; Min: with borderline SNT as true positives – Max: with borderline SNT as true negatives

Klaus et al. (Klaus et al., 2011) used the Immunozym All-species **FSME IgM** kit in an adapted version (Müller, 1997) due to the claimed higher sensitivity of the IgG+IgM protocol, since it should additionally detect early infections. This protocol was indeed tested with a small number of available cattle SNT-positives, but this did not seem to result in improved sensitivity, thereby supporting our initial hypothesis that IgG-testing for previous exposure is potentially more likely to have a higher overall sensitivity in field studies, at the inevitable cost of some false positives.

In addition, researchers will never know when the small window of opportunity of IgM detection (3-6 weeks post-infection) is exploitable in any field population (see Figure I-8). Most likely none of our cattle were experiencing an acute infection with IgM production (since all samples were collected during the winter months), and somehow the SNT-positives became falsely negative in the IgG+IgM protocol, making it even less sensitive compared to IgG-ELISA and SNT.

The Testline® EIA TBEV Ig® veterinary ELISA kit has been used once in a cattle, sheep and horse field study (Síkutova et al., 2009). The Enzygnost® kit has been used in dog studies twice (Csángó et al., 2004; Lindhe et al., 2009). As far as we are aware, none of the veterinary ELISA-tests in Table I-2, except the Progen® kit, were validated in veterinary species. No publications from the companies were found, therefore, it is difficult to say if any of the ELISA's has superior accuracy. A veterinary penside test may be handy in the future, but the Reagena® ReaScan TBE IgM (rapid test) was not intended and has not been evaluated for use in animals (Table I-2; Reagena, pers. comm., 2015).

Another WNV study has showed that with IDVET's ID screen West Nile competition ELISA kit, 67 horses were seropositive and all (100%) were in fact strong true TBEV SNT-positives, as confirmed in TBE-SNT (Rushton et al., 2013). Only 9 reactor horses showed a low grade reaction in the WNV- and USUV- SNT's (Rushton et al., 2013). This means that researchers studying WNV, USUV or LIV by ELISA in any animal species in Europe, should always check for TBEV in their differential of seropositive ELISA results.

In the future, more accurate TBEV-ELISA kits may be developed based on subviral particles (recombinant viral proteins) produced in mammalian cell cultures. Obara developed an in-house medical IgM/IgG ELISA based on FE-TBEV subviral particles, which showed higher sensitivity (94.1-98.8%) and specificity (100%: no cross-reactions with JEV) than a commercial medical test based on whole virions, when compared to SNT with a panel of human sera (Obara et al., 2006).

Ikawa-Yoshida developed a similar ELISA with subviral particles for rodent sera, and equally found increased accuracy (91.4% sensitivity and 100% specificity) opposed to one with recombinant E-proteins (77.1% sensitivity and 80.0% specificity), both compared to SNT (Ikawa-Yoshida et al., 2011).

VII.1.2.2 Confirmation Tests to rule out Cross-Reactions

Aspecific or false positive SNT-reactions cannot be 100% excluded in TBEV-negative samples 100%, especially at low dilution titers. Nonetheless, this remains a rare event and mostly in IgG kits (Klaus et al., 2014; Niedrig et al., 2007a; Niedrig et al., 2007b). Klaus et al. (2014) very recently reported rare cross-reaction in TBEV-SNT with homologous LIV in sheep. This is fairly unsurprising given the close phylogenetic and antigenic relationships between the viruses. Importantly, such cross-reactions were not found in other species infected with a variety of other flaviviruses at diverse dilutions/titers (Klaus et al., 2014).

Our studies equally documented some of the expected flaviviral **cross-reactions**, as one of the dogs reacted more strongly to LIV-HIT than to TBEV-SNT, and some of the wild boar showed a reaction in LIV-HIT and/or USUV/WNV-SNT. In flaviviral research, cross-reactions in ELISA kits are so common that we might hypothesize that almost "any" veterinary flaviviral ELISA kit could potentially be used as a screening test for TBEV-exposure.

Ultimately, these cross-reactions are only a real concern for laboratories in regions where multiple viruses could co-circulate (Klaus et al., 2014). This is currently highly unlikely in Belgium, where the presence of other flaviviruses has not been reported in any species during the studies performed and presented in this thesis (Beck et al., 2013; FASFC, 2011, 2012, 2013, 2014c, 2015a, b; Hubalek et al., 2014; OIE-WAHID, 2014), besides 2 single dead birds with USUV (Garigliany et al., 2014).

Despite being an absolute necessity, **SNT confirmation testing** against other possibly cross-reacting flaviviruses was not always straightforward. The selected SNT for a combination of species and flavivirus was not always readily available in Belgium/Europe (e.g. LIV/ USUV) or not validated for the species under study so that quality of the assays could not be guaranteed (e.g. WNV in wild boar). For the confirmation testing we preferably selected genetically related (LIV) and geographically relevant viruses (USUV, WNV, LIV: see Figure VII-1)(Beck et al., 2013). However, our sample panels came from non-target species for each respective test, e.g. wild boar, dog and cattle versus birds, horses, or small ruminants. Hence, control sera of the correct species-flavivirus combinations were often not available.

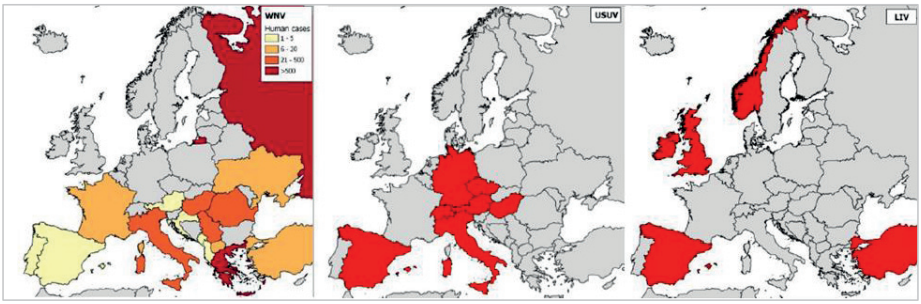


Figure VII-1: Flaviviruses geographically relevant to Belgium (present in neighboring countries).
WNV: West Nile virus; USUV: Usutu virus; LIV: Louping ill virus;
Selected from Beck et al., 2013.

Moreover, the SNT is a delicate test and poor quality samples necessitated kaolin sample treatments for the wild boar and cattle sera besides the usual pre-heating and treatment of stable cell cultures with antibiotics. Occasionally, we had to opt for "**second choice**" confirmation tests such as ELISA, HIT or IFAT. Nonetheless, despite the potential cross-reactions, in medical diagnosis HIT and IFAT are generally known as reasonably sensitive tests (Storch, 2007), that may also be specific in skilled hands through repetition and titer comparisons (Duscher et al., 2015b), and that may agree well with SNT (Litzba et al., 2014).

In the wild boar study, we tested the Euroimmun flaviviral IFAT-biochips (www.euroimmun.ch) for medical diagnostics (TBEV/WNV/JEV/YFV/DENV)(Litzba et al., 2014), after recalibration with specific primary/secondary conjugates and with porcine TBEV control samples obtained from the Friedrich Loeffler Institute (FLI). This IFAT confirmed only the three strongly SNT-positive/ELISA-positive wild boar and thus currently seems to be a less sensitive test than TBEV-SNT in wild boar.

VII.1.2.3 Test Validation

Due to the low accuracy characteristics of the veterinary TBEV-ELISA, it is recommended to validate these methods for each species tested. This can be done using standardized, highly positive as well as diluted control samples and ROC-analysis to adjust IgG cut-offs to obtain the DSe and DSp envisaged for specific surveillance purposes (rule-in or rule-out screening).

The few available veterinary commercial veterinary screening tests have most likely still been insufficiently validated and quality controlled by the manufacturers: there are no publications by the companies. In our studies, several positive samples were clearly missed by the IgG-ELISA and several false positives were equally observed in each species.

It is thus vital that at least during the first time that a test-protocol combination is used in TBEV-surveillance in a new species, attempts should be made to properly validate it before estimating seroprevalence or claiming freedom from infection. Some efforts have been done in domestic animals by Klaus and colleagues at FLI (Klaus et al., 2011; Klaus et al., 2010c) and in the present thesis, as described in Chapters III, IV, and V. However, TBEV remains a second priority pathogen in European veterinary and wildlife test validation projects (EWDA, 2015; Wildtech, 2010).

Another recurring issue in veterinary TBEV-ELISA testing is not excluding false negatives and/or positives. Many authors do not test all ELISA-positives in SNT, and most do not test any ELISA-negatives in SNT. However, at least 2 of the studies reported in the present thesis (cattle and boar: Chapters IV and V) have shown that the investment in confirmation testing of a considerable number of negatives must be made at least once per species, in order to evaluate the true DSe and DSp of the ELISA test protocol in the populations and species under study. This will allow adjustment of seroprevalence estimates for the imperfection of the screening test. Consequently, if we had retested all the ELISA-negatives in our dog study in SNT (Chapter III), perhaps more than one true positive dog would have been detected, and the canine population seroprevalence estimate may have been higher than 0.11%.

Lack of diagnostic TBEV test validation remains to our knowledge a large gap in current veterinary flaviviral diagnostics, which needs to be resolved at an international scale, through cooperation of veterinary (reference) laboratories with the ELISA producers, if veterinary sentinel surveillance is to be adopted to complement medical TBEV-surveillance in a standardized and quality-controlled way. International and standardized serum panels of diverse species-flavirus-combinations for veterinary proficiency testing are needed. The tests in use may benefit from no-gold-standard statistical evaluation (Klaus et al., 2011).

In any case, without robust confirmation, the current veterinary screening studies that are regularly published lose specificity and thus scientific value, as they actually may be saying more about “any apparent flavivirus prevalence” as opposed to the “true TBEV prevalence” in the population.

VII.1.2.4 Test Selection and Use

In general, it is known that measures of accuracy are not fixed indicators of a test performance (Šimundić, 2008) and that test accuracy in the field may be influenced by many factors, including population characteristics, genetic variation in the infectious agent, the sampling, storage and test methodologies and the population prevalence (Banoo et al.; Leeflang et al., 2009). With the ELISA accuracy results obtained during this thesis, it seems ill advised to start using any ELISA as a TBEV screening test in a low prevalence area at the fringe of the TBEV geographic distribution, at least not before some further international validation of these tests in multiple species.

The main reason for this is that Belgium and other low prevalence countries first have to be able to accurately map their endemic risk areas, as opposed to just estimating a true prevalence from an apparent ELISA-prevalence, to follow relative trends. For this risk assessment purpose, an unknown proportion of ELISA-false negatives may constitute a major problem. Clearly, this has never been an issue in the core areas of TBE(V) endemicity where the ELISA-tests were developed, as there the seroprevalence is usually quite high in one or more species, and clinical cases are much more common.

Therefore, the IFA and SNT are currently the most accurate veterinary serological tests for the Belgian situation, for either surveillance or diagnostic settings. Even when testing all sera with SNT/IFA tests is not sustainable in field screening, it should currently be best practice to test all ELISA-positives and -doubtfuls in SNT/IFA, together with a randomly selected sample of the ELISA-negatives. In the mean time, research projects should focus on continued (re-)validation and improvement of current ELISA's (e.g. sub-viral particles, study matrix and species effects on analytical and diagnostic sensitivity/specificity, sample preparation protocols, etc). The goal would be to obtain a more accurate and better characterized screening tool applicable to low prevalence settings for surveillance, risk assessment and trend watching purposes.

VII.1.3 Evaluation of Veterinary Sentinels (PhD Aim 4)

VII.1.3.1 The ideal TBE(V)-sentinel Species

The **ideal species** for TBEV sentinel surveillance should have an adequately limited home range in comparison to TBE focus size, which is often a few 0.5 - 1 km^2 (Dobler et al., 2011; Imhoff et al., 2015), should be available in large numbers, should be well dispersed in the surveillance area, and should show a long-lasting response after natural infection (Dobler, 2010; Gerth et al., 1995; Kunze, 2015). Additionally, (sero)prevalence should show a good spatial correlation with human TBE incidence, and frequent tick exposure/infestation is an advantage (Imhoff et al., 2015).

Considering the results in this thesis (Chapters III, IV, VI), a number of additional practical and epidemiological factors should equally be taken into account to set up an effective sentinel surveillance (Table VII-3), as these issues may severely limit certain aspects of the study, such as the diagnostic accuracy, the statistical power of the results, or the precision of any calculations (freedom/prevalence) and the potential to proceed with modelling or mapping exercises.

Table VII-3 Suitability criteria for TBEV sentinel surveillance	
Clinical Characteristics	- Clinical cases, viraemia and/or lasting antibody response - No flaviviral vaccination or exposure to other flaviviruses than TBEV - Tick exposure, good tick host and lack of preventive actions
Correlation with spatial human risk	- Useful proxy for human risk behaviour, mobility and travel - Spatial presence at national (NUTS 1), regional (NUTS 2), local (NUTS 3/4) - Suitable home range (km²) and representative - even distribution in the area
Epidemiological Parameters	- Knowledge of population size, density and sampling frame - Pre-existing surveillance for other pathogens: passive, active or targeted - Sample size sufficient for the surveillance purpose and design prevalence - Place on the iceberg: close to tip (clinical) or base (cycles)
Practical Parameters	- Organisations - Governments involved and available funding - Serum quality, volume and transport - Available flaviviral diagnostic tests for each species-virus combination

Table VII-3: Suitability criteria for TBEV sentinel surveillance.
Expanded from Dobler (2010), Gerth et al (1995), Kunze (2015), Imhoff et al. (2015)

VII.1.3.2 Experiences with and Suggestions for Dogs, Cattle and Wild Boar

The provincially selected private sentinel laboratories in Belgium were good contact points to construct an active sentinel surveillance component based on a **convenience** sample (leftovers from previous tests). This can be fine tuned by adding some more laboratories to covering the whole Belgian territory. At present, it would take a considerably larger effort to organize a nationwide **random** surveillance in this species, since national companion animal databases are not easily accessible for research. ID-Chip databases could be a starting point to obtain a sampling frame (e.g. Dogs: https://www.dogid.be/nl/home; Cats: http://www.idchips.com/nl/; Horses: http://www.cbc-bcp.be/identificatie/).

Aditionally, a very low exposure prevalence was found in the Belgian dogs tested (0.11%, n=880), which would seem to imply very large sample sizes needed to detect TBEV or to substantiate freedom (especially at a local level). On the other hand, some Belgian hotspots for ticks and Lyme disease such as Limburg and Luxembourg (Ducoffre, 2008b; WIV-ISP, 2011) were not covered in our dog study, which may have biased our results towards a low prevalence. Finally, we did experience difficulty in locating the actual exposure site for the SNT-seropositive animal, even with the detailed anamnesis that was obtained, e.g. travel history, tick bites and prevention and (flavivirus) vaccination status.

Therefore, dogs should currently preferably be involved in a **passive surveillance component**. Such a component would be easier to install, with the cooperation of the two university veterinary clinics in Belgium (in Liège and Gent). Severe neurological veterinary cases in dogs and horses are most likely to be seen by these referral centers, and they may already dispose of stored sera/CSF of interesting, undiagnosed aseptic meningitis cases (Dr. Cornelis I., , pers. comm., 2015).

In Belgium, **domestic cattle** are easy to sample as part of pre-existing veterinary surveillance programs (FASFC, 2014a). Since cattle and their movements are well-documented in the Belgian Sanitel Database (Ensoy et al., 2014; FASFC, 2016), serological reactions in cattle serum or milk should be the result of a verifiable and traceable exposure locality. Surveillance based on large scale existing national surveillance for cattle pathogens (FASFC, 2014a) would deliver steady and good quality cross-sectional and longitudinal sentinel data.

Random sampling is feasible and the resulting data are routinely registered by the Veterinary Authorities (FASFC) and readily accessible to government researchers. Despite the detectable prevalence in a similarly low range as the wild boar, the cattle titers were generally lower than the wildlife titers. The reason for this is currently unknown (e.g. lower tick exposure, test characteristics, low infection susceptibility).

Wild boar have been sampled in both Belgian regions before, to survey Aujeszky's disease (Czaplicki et al., 2006; Linden, 2005; Verpoest et al., 2014; Vervaeke, 2012), brucellosis (Gregoire et al., 2012; Linden, 2005; Vervaeke, 2012), classical swine fever (Linden, 2005; Mintiens et al., 2005; Vervaeke, 2012), bovine tuberculosis (Linden, 2005), and hepatitis E virus (Thiry et al., 2015). Despite the fact that wild boar are not distributed homogeneously throughout the whole Flemish territory (see Figure VII-2a), the results were still representative at the local level, as discussed in Chapter V (Roelandt et al., 2016).

The TBEV screening study in wild boar was easily attached to the existing yearly wildlife disease surveillance scheme in the Flemish communities. The field work and sample flow were pre-existing and well organised, however, background data on these wild animals is somewhat harder to obtain and was often incomplete. This also remains a convenience sample as opposed to the preferable first stage random sampling of spatial units (NIS/UTM), followed by a second stage hunter sampling of predefined sample sizes per demographic category (age – sex).

VII.1.3.3 Alternative Sentinels

In recent studies, **dogs and cats** have turned out to be cost-effective proxies/sentinels to target for human tick-borne disease risk assessments in (peri-)urban areas (Chomel, 2013; Jongejan and Uilenberg, 2013; Pfeffer and Dobler, 2013). Pets have certainly contributed to tick surveillance and identification of new pathogens and tick foci on a local scale (Beck et al., 2014; Claerebout et al., 2013; Eichenberger et al., 2015; Schreiber et al., 2014). **Horses** have been good sentinels and suitable for risk mapping in other countries (Imhoff et al., 2015; Janitza-Futterer, 2003; Klaus et al., 2013). They are worth considering for active (serological) and passive (clinical) sentinel surveillance folowing similar principles and remarks as for dogs.

In Wallonia and Flanders (2005-2015), **cervid samples** have been collected for paratuberculosis and tuberculosis surveillance (Linden, 2005), for surveys on Bluetongue virus, Schmallenberg virus and Hepatitis E virus (Linden et al., 2010; Linden et al., 2008; Thiry et al., 2015), and for serological screening for several ruminant pathogens (De Bosschere et al., 2006; De Craeye, 2012; Tavernier et al., 2015).

TBEV seropositivity was found in Wallonia (Linden et al., 2012) and in Flanders (Tavernier et al., 2015). Roe deer should be a priority for TBEV sentinel surveillance in Belgium, as they are key *I. ricinus* and TBEV hosts. They can serve for a randomized veterinary serosurveillance in forests to complement cattle surveillance in meadows. Most researchers have had good experiences with this species (see Tables I-3 and Chapter I). Furthermore, roe deer are more homogeneously spread throughout the whole Belgian territory: see Figure VII-2b (www.waarnemingen.be). A randomized sampling procedure would once again be preferable over risk-based or convenience sampling, to highlight as many risk areas as possible.

Despite the availability of existing surveillance networks for other (smaller) mammal and bird sentinel species, they should not be considered as a priority for TBEV sentinel surveillance. They may have too many drawbacks for TBEV surveillance such at large scale, such as small blood volumes for the necessary assays, suboptimal risk-correlation, less homogeneous spatial distribution or less sampling opportunities and large mortality.

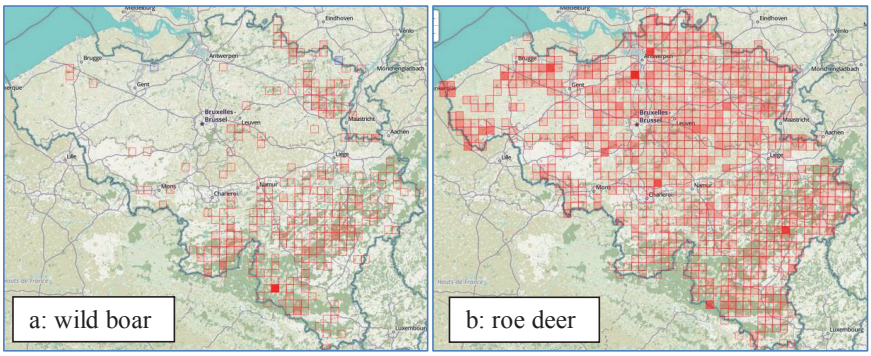


Figure VII-2a/b: Wild species sightings by the general public in Belgium (26/05/15 – 26/05/16).
a: Wild boar sightings (n=1235 animals in 262 UTM's); b: Roe deer sightings (n=2804 animals in 865 UTM's); UTM: universal transversal Mercator square unit (5 km), www. http://waarnemingen.be/

VII.2 CURRENT KNOWLEDGE GAPS AND PRIORITY ACTIONS (PHD AIM 5)

VII.2.1 PRIORITY 1: MEDICAL SURVEILLANCE AND AWARENESS

Belgium has always simply been assumed to be TBEV-free, though this was based on very little scientific evidence (Haglund et al., 2003). In 2016, despite five veterinary sentinel publications between 2010-2016, and despite multiple unexplained/inconclusive seropositive human cases, the medical world still considers TBEV as a non-endemic virus and of little importance to Belgium (Callens, 2016).

However, despite improvements in the diagnosis of viral encephalitis since the use of PCR on CSF to try and detect the more common viruses (Debiasi and Tyler, 2004; Jarrin et al., 2016; Parisi et al., 2016), the aetiology of up to 75% of aseptic/viral encephalitis and meningitis cases remains unknown around the world, even in 2016 (Donoso Mantke et al., 2008b; Frantzidou et al., 2008; Glaser et al., 2003; Harrell and Hammes, 2012; Jarrin et al., 2016).

Since 2010, there has been a TBEV National Reference Laboratory for Belgium, which is currently hosted by the Institute of Tropical Medicine (Antwerp). None of the Belgian samples submitted to the ITM between 2014-16 were positive in serology (n=40) or PCR (n=25), while occasional imported travel related cases (1 per year) continue to be diagnosed (Dr. M Van Esbroek, ITM, pers. comm., 2016).

This is more or less the expected number, considering ECDC and other data sources reported a total of only 38 travel-related cases for 2012 (Rendi-Wagner, 2004; Steffen, 2016).

Nonetheless, if 70% of TBE cases are symptomatic, the EU and Belgium should at least count n=127 and n=7 travel-related infections respectively. Considering the TBE-risk of 1 /10,000 for an unvaccinated tourist staying in highly endemic areas in Austria and applying this to total numbers of summer tourist overnight stays one should expect 60 clinical TBE case leaving Austria per year (Rendi-Wagner, 2004). There is still a significant amount of underdiagnosis and underreporting.

Moreover, TBEV does not feature in the regular diagnostic panel for locally acquired medical encephalitis (unless very clear anamnestic indications)(Jarrin et al., 2016; Parisi et al., 2016; Solomon et al., 2007) and medical surveillance has been very passive and limited (1 laboratory). The professional awareness in regards with prevention of travel related TBE cases is only beginning to rise now (ITM, pers.comm., 2016). For the clinician, it is important to try to establish an etiologic diagnosis in all cases of encephalitis/meningitis, even if there are no specific effective treatments. The identification of a specific etiological agent, such as TBEV, may still be important for the individual prognosis and counseling of patients and family members (Tunkel et al., 2008).

The Belgian veterinary sentinel studies (Linden et al., 2012; Roelandt et al., 2011; Roelandt et al., 2014; Roelandt et al., 2016; Tavernier et al., 2015), the Dutch ones (Jahfari et al., [submitted]; van der Poel et al., 2005) and the recent discovery of a new TBE-virus in the Netherlands on tick samples from September 2015 (Jahfari et al., [submitted]), should now prompt Belgian scientists and clinicians to reconsider this situation. The studies described and the evidence presented lead us now to reject the old hypothesis that TBEV is not present in Belgium and that only very few travel related cases are to be expected. It is internationally accepted now that even authochtonous human cases are merely the tip of the iceberg, and that TBEV shows an emerging character in several European countries (see Chapter I).

The Netherlands equally do not seem to have autochthonous TBE cases, but TBEV-infected questing ticks and seropositive sentinels are present within the Dutch territory. Clearly, enhanced medical surveillance and increased awareness among medical professionals are now absolute priorities for the Low Countries, necessary to minimize and assess any potential TBE risk to humans from this uncharacterised strain of TBEV and to guide prophylaxis and public health decisions and measures.

Additionally, medical surveillance may lead to explanations for the apparent mismatch between the veterinary findings and the lack of medical cases. Potential hypotheses to be explored may include: suboptimal diagnostic test quality, timing of (paired) sampling, insufficient testing by clinicians, lack of awareness, a large proportion of asymptomatic or clinically mild exposures, and presence of an atypical low-virulent TBEV virus.

TBE could be made notifiable, as in other European member states (ECDC, 2012, 2014). Next to the enhanced passive components it should include an active component, to increase detection sensitivity of the surveillance system (Hadorn and Stärk, 2008; Rodriguez-Prieto et al., 2014) and to improve usefulness, value and cost-effectiveness of the data (Thurmond, 2003). As suggested for veterinary surveillance, existing medical surveillance schemes and available ticks/sera/datasets should be exploited.

Since Lyme disease and TBE share a large part of their epidemiologic triad, vector, risk factors and cycle, there are obvious benefits of combining TBE surveillance/awareness campaigns with the newly developed Belgian Lyme Disease Surveillance and Awareness Strategy. This medical program (Lernout, 2016) also follows a pyramid/iceberg approach, as proposed by Braks et al (2014): see Figure VII-3. It will include data collection and awareness creation on tick bites and Lyme (https://tekennet.wiv-isp.be/)(Lernout, 2016).

This multi-component program will document tick bite GP consultations, include a large seroprevalence study in the general Belgian population (n=3,215 from 2013-14) and analysis of longitudinal seropositivity trends (sentinel laboratories). Finally, a project will assess the health burden and cost of Lyme disease (Lernout, 2016), which currently causes a median of 1,132.5 hospitalized cases per year in Belgium (2003-2012) (Bleyenheuft et al., 2015).

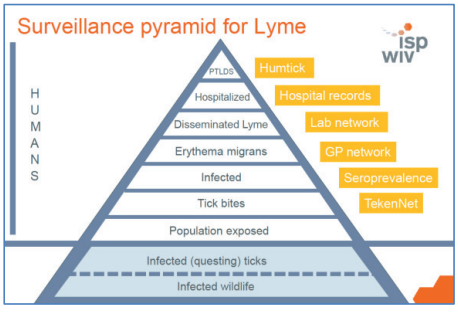


Figure VII-3: Belgian Medical Surveillance Strategy for Lyme Disease.
As example for TBE medical surveillance (Braks et al., 2014; Lernout, 2016)

VII.2.2 Priority 2: Virological Research

The probability to detect TBEV via direct methods such as isolation and PCR, in ticks, humans or animals, is low given the currently available methods and low prevalence setting (see Chapter I). Due to this low detection probability and the considerable resources needed to collect sufficient ticks/samples to obtain confident results, scientists are often not activated to conduct such surveillance activities in low prevalence areas (Stefanoff et al., 2013). Such practical and financial objections have until now hampered efforts towards direct testing in Belgium, but also in other EU member states.

TBEV does not often cause epidemics with high case loads, as might be the case with other vector-borne emerging diseases (e.g. Bluetongue virus or Chikungunya). Occasionally, one may be confronted with a food-borne outbreak with a few dozen cases, e.g. Košice, Slovakia, May-June 2016 (KICM, 2016). This flavivirus remains mostly submerged in its tick-host cycles within largely unaffected populations of diverse wild/domestic species (Figure I-1, I-2). It causes only short viremia and the majority of infections are asymptomatic (Figure I-7).

This makes it very difficult to catch the virus "in action" and the international scientific community still rarely succeeds in isolating TBEV-strains from known human/veterinary cases, or from hosts even in known highly endemic areas. Well characterized and fully sequenced TBEV isolates are scarce throughout the entire Eurasian endemic zone (Belikov et al., 2014; Bertrand et al., 2012; Formanova et al., 2015). Some of the characterized TBEV strains were isolated from ticks or rodents, and rarely from a human case (Fajs et al., 2012; Golovljova et al., 2004; Leonova et al., 2013; Süss, 2011; Wallner et al., 1996).

TBEV is capable of **evolution**, mutation and recombination when passaged in the lab through different hosts (Bertrand et al., 2012; Kaluzova et al., 1994; Romanova et al., 2007), in the field throughout the Eurasian continent (Pogodina et al., 2007; Zanotto et al., 1995), and also at the biogeographic edges of its distribution, where it is subjected to a number of ecological constraints (Carpi et al., 2009; Hubalek et al., 1995). At the edges of the European distribution, the related louping ill virus (LIV), Spanish sheep encephalitis virus (SSEV), Greek goat encephalitis (GGEV) virus and Turkish sheep encephalitis virus (TSEV) (Hubalek and Rudolf, 2012; Hubalek et al., 2014), and the in 2015 characterised Spanish goat encephalitis virus (SGEV) virus are present (Mansfield et al., 2015).

Very recently (June 2016), a Dutch-Belgian research team successfully detected TBEV-viral RNA from an unknown TBEV strain (Jahfari et al., [submitted]). This was in two ticks collected in September 2015, obtained in a forested area where 6 roe deer sera from 2010 were found seropositive. The Dutch isolates were found to cluster within the TBEV species complex, but not within the three established TBEV subtypes (W-S-FE) nor within the LIV cluster, implying that it concerns a novel subtype (Jahfari et al., [submitted]).

So far, the TBE-virus has not been amplified or isolated yet in Belgium. The study on 13 wild boar tonsils (all negative) was the first published attempt (Roelandt et al., 2016). Secondly, during 2014-2015, the WIV-ISP has executed a field study in rodents in some of the communities where the Belgian TBEV-seropositive cattle were found (Roelandt et al., 2014). Several authors have indeed advocated rodents (serum, brain, spleen) for attempts to find TBEV, and capturing them in areas highlighted by veterinary serosurveillance is current good practice.

Nonetheless, so far the Belgian rodents have been SNT-negative (n=0/173) and PCR-negative (n=0/308) (Dr. Brochier B., pers. comm., 2016). This could be due to a number of factors, such as large rodent turnover so they remain only seropositive for a short time, low TBEV tick-prevalence and non-viremic transmission, or just bad luck with the location of the sites, as endemic foci can be quite small. The sentinel and tick research should continue at least until a Belgian TBEV strain is characterized, as this strain may very well be an atypical strain as the one found in the Netherlands.

VII.2.3 Priority 3: Risk Assessment based on One Health Surveillance

VII.2.3.1 One Health Surveillance

In a globalized world with increasing numbers of emerging diseases, an interdisciplinary so-called ”one health” approach is indispensable for the prevention and control of vector-borne zoonoses, such as TBE (Braks et al., 2014). This approach leads to better preparedness and contingency planning, more effective surveillance and control systems, increased health equity and improved sharing of logistics and costs (Leach and Scoones, 2013; Obsomer et al., 2013). In addition, knowing the precise location of infection risk can lead to better targeted prevention and control measures, control (Obsomer et al., 2013).

Medical surveillance and awareness should now be a critical part the Belgian TBE(V) surveillance program, given the potential clinical zoonotic importance of this pathogen, even if afterwards it turns out to be non- or low- pathogenic for humans. On the other hand, veterinarians also have a role to play, since sentinel species help the scientific community to explore the bulk part of TBE epidemiology, to perform risk assessment, to define risk areas and to guide researchers to the TBE-virus itself.

In general, Belgium should still first focus on the **randomized national** (regional) surveillance components, certainly in humans but still in animals, as currently the general picture is still very incomplete. Many communities and some Belgian provinces have not been sufficiently covered. This can include both active as passive surveillance components, involving serological, clinical syndromic and virological follow-up, which can be organized through the existing networks.

Humans, cattle and roe deer are the most suitable species for a national active component. A randomized survey design is to be preferred in all species if serology data are to be used for prevalence estimation, risk mapping and modeling afterwards. Any clinical cases detected in enhanced national passive surveillance in sentinel hospitals and practices should be fully traced and a very detailed exposure and vaccination anamnesis should be obtained.

Each time that a national/regional surveillance component has been analysed for a certain area (province, community), the competent governments can move their attention to highlighted areas for **risk-based local** surveillance. This includes trying to find TBEV in viremic reservoir rodents or infected ticks as well as continued serology in humans and sentinel animals in specific, highlighted areas. This is a second phase surveillance in a low prevalence situation, as it will generally require much more resources to obtain results: a virus isolate, RNA or (a)symptomatic cases. The endemic cycle of TBEV may be very localized with low tick prevalence. Therefore, it is advised to implement this research at the local level (community, NIS, postal code) or in a natural landscape unit (e.g. UTM square, forest, park, field, garden) with known seropositive animals or to collect ticks directly off hosts.

VII.2.3.2 Modelling and Mapping

Up till now, real case maps or spatial predictive risk modelling were considered unfeasible for TBEV in Belgium, in particular because of lack of human case/exposure data, randomised serological sentinel data or tick TBEV-prevalence data covering the whole country. There are no autochthonous clinical human/animal cases yet, and the recording of numbers of tick bites per municipality has only started at a national level in 2015: see Figure VII-3 (Lernout, 2016). Until now, there are no published studies on TBEV-prevalence in Belgian ticks. The case data obtained in this thesis was based on sentinel studies that were differently designed for species playing different epidemiological roles. As such, the obtained data are not necessarily representative for the whole Belgian territory or for other species. Indeed, the cattle and wild boar data (bottom iceberg) cannot easily be combined with human and canine seroprevalence data (tip iceberg).

The combined cattle and wild boar seroprevalence data alone ($Animals_{tested}$=888 with $Animals_{pos}$=33=3.72%; Cattle $herds_{sampled}$=41 with $herds_{pos}$=10; $NIS_{sampled}$=45 with NIS_{pos}=15; NIS: National Institute of Statistics code for Community/Municipality) are not sufficient to serve as case data in a multivariate Poisson model with continuous/categorical predictor variables. Firstly, the positive NIS, herd and animal sample sizes are insufficient, leading to a potential lack of power (type II errors) (Dohoo et al., 2009a), biased estimates and variance inflation (Dohoo et al., 2009d).

Equally, we would risk false positive regression coefficients (type I errors) by multiple comparison errors (Dohoo et al., 2009c). A Poisson or negative binomial distribution may approximate our data (Figure VII-4), however, due to likely overdispersion ($s^2>\bar{x}$) and excess zeroes (n_{zero}=30/45=66% of the NIS, the zero-inflated model may be the best candidate, as zero counts can then be generated both in infected as non-infected communities or herds (Dohoo et al., 2009e).

However, the sample size of positive and negative NIS/herds should ideally still be at least 10*(k+1) (k=3-6: the no. of estimated predictors in the final model), in order to avoid the issues listed above and lack-of-fit or non-convergence of the model (Dohoo et al., 2009d; SAS, 2008), implying a much larger study containing **at least 40-70 positive NIS** (Belgian communities) or herds, which is currently not available (Figure VII-4).

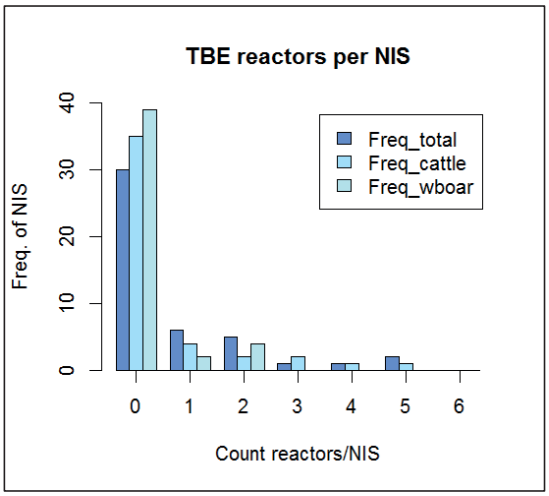


Figure VII-4: Number of TBE reactors per sampled NIS.
Bar plot of 0-6 reactors per NIS. NIS: National Institute of Statistics code for Community/Municipality.

As discussed in Chapter VI and listed in Annex VI.4, there are a multitude of potential TBEV risk factors to choose from to perform a modelling or mapping exercise. They are not always consistently significant throughout studies. In Chapter VI, we selected data that were available in Belgium on the most mentioned TBE risk factors: large hosts, *Ixodes* ticks, human proximity, forests, and temperature. This covered the 3 main areas of risk as well as meteorology.

Many other layers can of course be selected depending on data availability and the search for suitable Belgian datasets at sufficient spatial resolution continues. However, the final model/map should not contain too many predictive variables, for the same epidemiological reasons as mentioned above for the case data: statistical power, model convergence, false positive effects. So, if many datasets on the potentially confounding and interacting risk factors become available, variable selection methods (e.g. forward/backward selection; regression tree pruning; sensitivity analysis) should be used to select the best subset of TBEV risk predictors for the Belgian situation.

VII.2.3.3 European Meta-Analysis

Researchers may consider a renewed TBE-meta-analysis for the whole of Europe, in the spirit of Randolph and Green (1999) and Randolph and Rogers (2000). Currently, some of the national/regional modelling papers still contradict each other on the relative importance of certain landscape, host or climatic risk factors. A quantitative (Bayesian) meta-analysis could examine the heterogeneity and publication biases in the existing studies and may provide the scientific community with more precise, powerful, valid and consistent estimates of effects and interactions than any single study could do (Lean et al., 2009; Sargeant et al., 2014; WHO, 2015).

This would involve (re-)analyzing existing datasets from known endemic and free areas in Belgium and from neighboring EU countries in multi-level models. Through a pooling of international clinical and serological case data from humans, domestic/wild animals or tick data collected at the community level (NUTS4) or at least regional level (NUTS3), generalized regression coefficients for local factors and interactions with higher level variables (climate) could be determined.

However, this approach would have to involve a multi-year international project with data exchange between research groups and the final results may still not be completely tailored to the local situation (i.e. extrapolated) or the results may only be delivered after the first Belgian TBE case(s). Therefore, this should be a second choice approach from the Belgian point of view: it remains preferential to collect sufficient Belgian medical and virological data to enable risk assessment together with the available risk factor data.

VII.3 CONCLUSIONS

In this doctoral work, serological evidence was obtained in favour of TBEV-presence in Belgium. Serological surveys were performed in three relevant veterinary species: dogs (Chapter III), cattle (Chapter IV) and wild boar (Chapter V). These studies have confirmed the exposure of Belgian animals to TBEV, based on the use of the serological gold standard test (SNT). Exposure may have occurred in Belgium (cattle, wild boar, dog?) or abroad (wild boar, dog?). The indirect serological evidence was strong, with high SNT titers in some animals. In addition, sera from positive cattle showed protection in a mouse bioassay (TBE challenge experiment).

An extensive literature search (Chapter I) confirmed that indirect serological evidence is in most cases the only suitable and practical evidence for TBEV presence/absence substantiation, especially in a low prevalence and national surveillance setting.

A first attempt was conducted to map the geographical distribution of the TBEV seropositive communities against the known spatial data on known TBE(V) risk factors in Belgium (Chapter VI). This simplified exercise showed qualitatively and statistically that combined seropositivity of cattle and wild boar corresponds to the areas with a higher number of risk factors. In the future, more data should be collected on a national or regional scale to enable true predictive modelling and spatial mapping.

In order to evaluate the current serological diagnosis of TBE in veterinary species (Chapter VII), a commercial ELISA screening test (Progen) was evaluated in the selected sentinel species (Chapters III, IV, V). Cross-reactivity testing and test validation in general need additional research attention in the future. As long as veterinary ELISA's are internationally not well validated and accuracy improved in cooperation with the producers, SNT and IFA are recommended for TBEV diagnosis and surveillance in low prevalence situations.

The selected sentinel species from the previous chapters were evaluated based on criteria from the literature and on our survey experiences. This was done with a view to determine their suitability for ongoing TBEV-sentinel surveillance (Chapter VII). The three evaluated sentinel species were each found to be useful for at least one specific purpose, e.g. for local, regional, or national surveillance; for passive or active surveillance; for risk-based or random surveillance; for clinical, serological or virological surveillance.

With the knowledge that TBEV is present in Belgium, medical surveillance should be enhanced to minimize risk and to explain the apparent mismatch between veterinary sentinel studies and the lack of medical cases in the Low Countries. The TBE-virus needs to be found in ticks, clinical cases or viremic hosts and fully characterized. Finally, a broad one health surveillance approach was suggested to allow TBE risk modelling/mapping and/or a European TBE meta-analysis in the future (Chapter VII).

CHAPTER VIII SUMMARY OF THE THESIS

Summary

VIII.1 INTRODUCTION – AIMS

The Western subtype Tick-borne encephalitis virus (TBEV) is the most important, highly pathogenic, neurotropic arthropod-borne flavivirus in Europe, and is carried by *Ixodes ricinus*. Tick-borne encephalitis (TBE) has become a considerable public health risk in many European countries with currently on average 3,000 hospitalized cases per year, and with long-term sequelae and disability in many patients.

Recent increases and fluctuations in human incidence in Central and Eastern European countries and the emergence of the disease in Scandinavia and France, have sparked international concern and research. TBE is also emerging in Europe's canine and equine population, and the numbers of clinical cases are expected to increase, as travelling and veterinary awareness increase.

The literature review in the introduction aims to highlight important features of TBE(V) epidemiology, the clinical course, the diagnostics and the surveillance possibilities for this tick-borne flavivirus. There are no confirmed autochthonous Belgian TBE cases. However, there are strong suspicions in the medical community (pers. comms.) and favorable environmental and (a)biotic conditions are present. Hence, it was discussed why Belgium is at risk for TBE(V) emergence and why active and national veterinary surveillance should be of benefit to public health in addition to the existing medical surveillance.

The aims of this PhD were multiple:

1. To establish veterinary serological evidence pro or contra TBEV presence in Belgium, based on studies in key species relevant for TBEV epidemiology and surveillance

2. To map the geographical distribution of the samples and seropositive cases versus a number of known TBE risk factors

3. To evaluate an ELISA first-line test for veterinary screening based on the gold standard seroneutralisation test

4. To evaluate the selected sentinel species (domestic and wild) regarding their suitability for ongoing veterinary TBEV surveillance based on experiences and literature

5. To identify remaining knowledge gaps and suggest priority actions for the future

VIII.2 CANINE SEROLOGY STUDY

Tick-borne encephalitis virus (TBEV) is an emerging tick-borne viral infection of dogs in Europe. Therefore, a commercial enzyme-linked immunosorbent assay (Progen® ELISA) was adapted for the detection of TBEV-specific IgG-antibodies in canine sera. In 2008, serum samples of Belgian dogs were obtained from three diagnostic laboratories located in Northern (n=688) and Southern Belgium (n=192), and their distribution was mapped.

ELISA-positive (n=2), (near-)borderline (n=8) and some negative (n=10) samples were subjected to the gold standard seroneutralisation test (SNT), based on the TBEV rapid fluorescent focus inhibition test protocol (RFFIT). One dog was confirmed TBEV seropositive in TBEV-SNT. Nine samples giving a borderline or positive TBEV ELISA result (including the SNT-positive sample) and two of the negative samples were further screened for West Nile virus (WNV) and were found negative. Five samples from which sufficient serum remained, were then additionally tested by LIV HIT: one borderline sample tested LIV antibody positive (titer 1:160), which may have been an LIV- or a TBEV-reaction.

We remarked that the cut-off for the Progen® ELISA IgG protocol could possibly be decreased to to increase the sensitivity of the test for canine sera. It was concluded that it would be prudent to further validate and standardize this ELISA test for estimating prevalence of infection or exposure to TBEV in several species, including dogs.

The clinical history of the seropositive dog could not explain beyond doubt where and when TBEV infection was acquired (in Belgium or abroad – recently or 7-8 years before testing). Further surveillance was found necessary to determine whether this dog represented a single travel-related case or whether it represented an early warning of a possible future emergence of TBEV.

VIII.3 BOVINE SEROLOGY STUDY

The risk of TBEV-introduction into Belgium remains high and domestic animals can serve as excellent sentinels for TBEV-surveillance, in order to install an early warning surveillance component for this emerging zoonotic disease of public health importance. In a targeted, risk-based and cross-sectional sampling design, serological screening was performed on Belgian cattle (n=650), selected from the 2010 Belgian national cattle surveillance serum bank.

Summary

All samples were subjected to a commercial ELISA (Progen®) and to the gold standard TBEV seroneutralisation test (SNT), based on the rapid fluorescent focus inhibition test protocol (RFFIT). Seventeen bovines were seropositive (titer >1/15) and six had borderline results (1/10 < titer < 1/15). The accuracy of the SNT was confirmed with cross-reactivity WNV and Rabies virus SNT, ELISA-tests and in a mouse inoculation experiment/test.

The IgG protocol of the Progen ELISA® seemed to have an extremely low relative DSe in cattle, combined with a fairly reasonable relative DSp. The precision, predictive values, Cohen's kappa and Youden index, also followed the same trends, indicating an overall low capacity of this test/protocol to distinguish and correctly classify TBEV seropositive and negative cattle. When inspecting the cattle ROC curves (AUC=54%), we felt that no big improvement could be made to this particular protocol by changing the cut-off in this species. A generally used formula for a calculated cut-off value ($c = \mu_{neg} + 2*SD_{neg}$) would still have led to a large amount of miss-classification.

The overall bovine TBEV-seroprevalence in the targeted area was estimated between 2.61 and 4.29% based on the SNT results. This confirmed the presence of infected foci in the Eastern parts of Belgium for the first time. Further surveillance in cattle, other sentinels, ticks and humans at risk was recommended to further determine the location and size of endemic foci and the risk for public health.

VIII.4 WILD BOAR SEROLOGY STUDY

In the frame of a Flemish wildlife surveillance in 2013, a serological screening was performed on sera from Flemish wild boar (*Sus scrofa*; n=238) in order to detect TBEV-specific antibodies. These sera were taken throughout the whole Flemish wild boar population range. All samples were subjected to gold standard TBEV seroneutralisation (SNT). Seven wild boars were seropositive and showed moderate to high SNT-titers - three had borderline results. Seroprevalence was estimated around 4.20% (95%CI: 1.65-6.75%). Other Flaviviridae (Classical Swine Fever, West Nile Fever, Louping Ill viruses) were excluded and thirteen available tonsils tested negative in TBEV RT-PCR.

Summary

The test characteristics of The Progen® TBEV-ELISA were assessed against the gold standard results. The IgG protocol showed low diagnostic sensitivity and good diagnostic specificity (DSe: 40-57% and DSp: 91-92%). ELISA agreement with the SNT was judged "slight to fair". ROC-analysis showed that for early detection screening purposes the ELISA cut-off might be placed as low as 35 Vienna-units: this would result in improved DSe (70-71%) at the cost of DSp (64.04-69.74%).

This study showed the presence of TBEV-specific antibodies in wild boar and potential TBEV-foci in Flanders (both in Limburg and West Flanders). Ongoing wild boar surveillance could serve as a local or regional sentinel warning system for public or human health prevention. Additional active surveillance and direct testing are now recommended to attempt virus detection and to further determine the characteristics of endemic foci, while continued passive medical and veterinary surveillance is indicated to monitor the potential risk for Belgian public health.

VIII.5 MAPPING AND MODELLING BELGIAN TBE DATA

There are several types of maps and models used in TBE research: prevalence and sampling maps, predictive risk maps, spatial and predictive models. Since Belgium currently has no confirmed human TBE cases, we descriptively mapped the sampling datasets and the seropositive sample results from the three veterinary sentinel studies performed in this PhD. Besides the veterinary results obtained, nine inconclusive but suspected human patients were added, that reacted negative in tests for neuroborreliosis (Lyme disease), positive in TBEV-SNT and negative in IgM TBEV-ELISA.

Using several host, vector, landscape and meteorological spatial distribution layers, it was attempted to map TBE(V) risk factors for Belgium. Due to the current lack of sufficient case data for predictive modelling, a first and simple approach was used, by selecting the "extreme 20%" quantiles for each of the TBE risk factors and by summing the risk factors per Belgian surface pixel with equal weights. Despite the simple and crude approach, this indicated in a qualitative way, that the seropositive animals and humans are found in the as such defined TBE "at risk" zones. The co-feeding paradigm and LST slopes/cycles should be re-investigated in the Low Countries.

VIII.6 DISCUSSION

VIII.6.1 AIMS AND FINDINGS

The findings of this PhD were discussed following the PhD Aims:

PhD Aim 1: TBEV has been present in Belgium since at least 2010. The current evidence is indirect (5 veterinary sentinel studies Belgium), but internationally acceptable;

PhD Aim 2: the samples and results were mapped and a risk factor map was constructed based on known TBEV risk factor layers. The overlay of the seropositive cattle/wild boar matched with pixels with qualitatively more factors;

PhD Aim 3: It was discussed what would be needed for better validation and selection of TBEV diagnostic tests. International cooperation with ELISA producers and collection of more reference samples for multiple species would be beneficial here. For now, SNT and IFA are the most accurate tests for veterinary use in Belgium;

PhD Aim 4: The selected sentinel species were evaluated for suitability criteria and were found suitable for specific purposes. Other sentinels were suggested, cervids are to be a priority.

PhD Aim 5: Identifying knowledge gaps and suggesting priority actions (see VIII.6.2.).

VIII.6.2 GAPS AND ACTIONS

Medical surveillance is now an absolute priority, to elucidate the apparent discrepancy between the veterinary findings and a lack of medical cases. The amount of aseptic meningitis encephalitis cases with etiological diagnosis needs to increase by including TBEV in the diagnostic panel. Exposure and infection prevalence should be assessed in a national serosurvey. The necessary TBE surveillance components (active and passive) could easily be attached to the Belgian Lyme disease and tick bite surveillance and awareness strategy components.

Direct evidence of an atypical TBE-virus was recently discovered in the Netherlands (Jahfari et al., [submitted]). Despite unsuccessful attempts in Belgium so far (wild boar tonsils - rodents), this search must continue in clinical cases, ticks and hosts especially in areas highlighted through randomized national surveillance. The Belgian TBEV-strain needs to be fully characterized and its virulence assessed.

Summary

An interdisciplinary One Health approach to TBE will be indispensable to assess and map the risk of this emerging pathogen. Surveillance should include national randomized and local risk-based studies on different target species. The data from these studies will allow detection of the virus and spatial predictive (risk/factor) modelling, which is still unfeasible at this time. A renewed European meta-analysis for TBE may be indicated.

VIII.7 CONCLUSIONS

TBEV seems to have been present in Belgian veterinary sentinel species from 2010 onwards. The commercial ELISA test was evaluated for screening but the SNT and IFA have superior accuracy. Simultaneous extensive test validation for different species and flaviviruses remains a challenging and urgent task. The three selected sentinel species (dogs, cattle and wild boar) were found suitable for different types of complementary veterinary TBE surveillance. The medical community should now increase TBE surveillance to assess the risk. The virus strain needs to be found and assessed. For this purpose ticks, cervids (roe deer) and rodents need to be investigated. One health interdisciplinary TBE(V) surveillance should lead to sufficient data collection for predictive modelling and true risk mapping in future studies.

RELEVANT BIBLIOGRAPHY

PEER REVIEW AND GREY LITERATURE

Roelandt S., Heyman P., Tavernier P., Roels S. (2010). Tick-borne encephalitis in Europe: review of an emerging zoonosis. Vlaams Diergeneeskundig tijdschrift 75(1): 23-31. **(Impact 0.417)**

Roelandt S., Heyman P., Filette M.D., Vene S., Van der Stede Y., Caij A.B., Tavernier P., Dobly A., De Bosschere H., Vyt P., Meersschaert C., Roels S. (2011). Tick-Borne Encephalitis Virus Seropositive Dog Detected in Belgium: Screening of the Canine Population as Sentinels for Public Health. Vector Borne Zoonotic Dis. 11(10): 1371–1376. **(Impact 2.298)**

Roelandt S., Suin V., Riocreux F., Lamoral S., Van der Heyden S., Van der Stede Y., Lambrecht B., Caij B., Bochier B., Roels S. (2014).Van Gucht S. Autochtonous Tick-Borne Encephalitis Virusseropositive cattle in Belgium: A risk-based targeted serological survey. Vector-Borne Zoonotic Dis 14(9): 640-647. **(Impact 2.298)**

Roelandt S., Suin V., Riocreux F., Lamoral S., Van der Heyden S., Van der Stede Y., Lambrecht B., Caij B., Bochier B., Roels S., Van Gucht S. (2014). Oral Presentation: Autochtonous Tick-Borne Encephalitis Virus seropositive cattle in Belgium: A risk-based targeted serological survey. Parasites and Vectors 2014, 7(Suppl 1): O35. **(Impact 3.43)**

Roelandt S., Suin V., Van der Stede Y., Lamoral S., Marché S., Tignon M., Saiz J.-C., Escribano-Romero E., Casaer J., Brochier B., Van Gucht S., Roels S., Vervaeke M. (accepted 2016). First Serological TBEV screening of Flemish wild boar. Infection Ecology and Epidemiology.

Roelandt S., Suin V., Van Gucht S., Van der Stede Y., Roels S. Comparative TBEV surveillance in Belgium during 2009-2015: Experiences regarding diagnostic tests and surveillance designs.Review Paper requested by Veterinary Sciences (In preparation - to be submitted in 2016). MDPI Publishers - http://www.mdpi.com/journal/vetsci

Roelandt S., Ducheyne E., Suin V., Van Gucht S., Roels S., Van der Stede Y. Qualitative spatial assessment of human TBE risk factors in Belgium. Archives of Public Health (In preparation – to be submitted in 2016). BioMed Central - Springer - http://archpublichealth.biomedcentral.com/

Roelandt S., Riocreux F., Van der Heyden S., Suin V., Lamoral S., Brochier B., Van Gucht S., Caij A.B., Lambrecht B., Roels S., Van der Stede Y. (2013). Type II evaluation of commercial diagnostic reagents: use of RFFIT (SNT) and FSME "all species" - antibody ELISA® during a risk-based screening for tick-borne encephalitis (TBE) Antibodies in bovine sera. Report submitted to FASFC on 13/09/2013.

Roelandt S., Heyman P., Filette M.D., Vene S., Van der Stede Y., Caij A.B., Tavernier P., Dobly A., De Bosschere H., Vyt P., Meersschaert C., Roels S. (2012). Tick-Borne Encephalitis Virus Seropositive Dog Detected in Belgium: Multi-species Screening of Sentinels for Public Health - Tick-Borne Encephalitis Virus Seropositieve Hond Gedetecteerd in België: Multi-species Screening van Sentinels voor de Volksgezondheid - Chien Séropositif pour le Virus de l'Encéphalite à Tiques Détecté en Belgique: Dépistage Multi-Espèce de Sentinelles pour la Santé Publique. FAVV Labinfo 8: p. 23-26.

Roelandt S., Heyman P., Filette M.D., Vene S., Van der Stede Y., Caij A.B., Tavernier P., Dobly A., De Bosschere H., Vyt P., Meersschaert C., Roels S. (2011). First tick-borne encephalitis virus (TBEV) seropositive dog discovered in Belgium: Screening of the canine population as sentinels for public health Scientific Report 2009/2010. Veterinary and Agrochemical Research Centre. pp.53-55.

Suin V., Roelandt S., Lamoral S., Brochier B., Van Gucht S., 2013. Sentinel Surveillance for tick-borne encephalitis virus in belgium: a one-health success story. Scientific Brochure WIV-ISP 2012-2013. pp. 6.

RELEVANT ORAL PRESENTATIONS AND POSTER-ABSTRACTS

Poster-Abstract at 5th BWDS Symposium, 16/10/2009, Brussels, Belgium. Tick-borne encephalitis (TBE): screening of the Belgian dog population as sentinels. Abstractbook p.36.

Poster-Abstract at SVEPM Annual Meeting 24-26/3/2010, Nantes, France. Tick-borne encephalitis (TBE): First seropositive dog detected during screening of the Belgian canine population as sentinels.

Poster-Abstract at MedVetNet conference, 24-25/06/2013, Copenhagen, Denmark. Tick-borne encephalitis virus-seropositive cattle in Belgium: a risk-based screening for (TBEV) antibodies in bovine sera. Abstract book p. 49.

Oral Presentation (by Marcella Mori) at EurNegVec conference, 8-11/04/2014, Cluj-Napoca, Romania. Autochthonous Tick-borne encephalitis virus (TBEV) – seropositive cattle in Belgium: A risk-based targeted serological survey. Abstract book p.39.

Poster-Abstract at AESA-FSVEE Joint Study Day 2014, 28/10/2014, Brussels, Belgium. Autochthonous Tick-borne encephalitis virus (TBEV) – seropositive cattle in Belgium: A risk-based targeted serological survey. Abstract book p. 51.

Poster-Abstract at FSVEE Study Day 2014, 30/10/2015, Brussels, Belgium. TBEV seroprevalence and test accuracy in Flemish wild boar. Abstract book p.71

Oral Presentation at BELVIR Meeting, 08/12/2014, Brussels, Belgium. Autochthonous Tick-borne encephalitis virus (TBEV) – seropositive cattle in Belgium: A risk-based targeted serological survey. Abstractbook p. 31.

Oral Presentation at 6th BWDS Symposium, 16/10/2015, Brussels, Belgium. TBEV seroprevalence and test accuracy in Flemish wild boar. Abstract book p. 19.

REFERENCES

Åblad B. (2007). TBE hos entvåårig hund i Västra Gätaland. Svensk Vet Tidn 59(13).

Acevedo P., Gortazar C. (2014). European lagomorphs. In: Cooke B. and Tizzani P. (Eds.) Network for wildlife health surveillance in Europe Species Card: pp. 4. http://www.aphaea.eu/sites/default/files/card_extern/aphaea_sc_lagomorphs_050315.pdf.

Acevedo P., Quiros-Fernandez F., Casal J., Vicente J. (2014). Spatial distribution of wild boar population abundance: Basic information for spatial epidemiology and wildlife management. Ecol. Indic. 36: 594-600.

Acevedo P., Vicente J., Hofle U., Cassinello J., Ruiz-Fons F., Gortazar C. (2007). Estimation of European wild boar relative abundance and aggregation: a novel method in epidemiological risk assessment. Epidemiol Infect 135: 519-527.

Achazi K., Ruzek D., Donoso-Mantke O., Schlegel M., Ali H.S., Wenk M., Schmidt-Chanasit J., Ohlmeyer L., Ruhe F., Vor T., Kiffner C., Kallies R., Ulrich R.G., Niedrig M. (2011). Rodents as sentinels for the prevalence of tick-borne encephalitis virus. Vector Borne Zoonotic Dis 11: 641-647.

AEP. (2014). Chronic Wasting Disease: Attention hunters ! Alberta Environment and Parks - Fish and Wildlife Diseases. http://esrd.alberta.ca/fish-wildlife/wildlife-diseases/chronic-wasting-disease/default.aspx.

AGIV. (2009). Geo-Vlaanderen Natura2000. Agentschap voor Geografische Informatie Vlaanderen.

Alekseev A.N., Burenkova L.A., Vasilieva I.S., Dubinina H.V., Chunikhin S.P. (1996). Preliminary studies on virus and spirochete accumulation in the cement plug of ixodid ticks. Exp Appl Acarol 20: 713-723.

Alekseev A.N., Dubinina H.V. (2002). Stability of parasitic systems under conditions of anthropogenic pressure. In: K.V. Galaktionov (Ed.) Contributions from the Zoological Institute. Russian Academy of Sciences 6: http://www.zin.ru/contributions/n06/.

Alexander N.S., Massei G., Wint W. (2015). The European distribution of Sus scrofa. Model outputs from the project described within the poster - Where are all the boars? An attempt to gain a continental perspective. Retrieved 14:25, Oct 26, 2015 (GMT) from http://dx.doi.org/2010.6084/m2019.figshare.1502662

Alexander N.S., Morley D., Medlock J., Searle K., Wint W. (2014). A First Attempt at Modelling Roe Deer (Capreolus capreolus) Distributions Over Europe. Open Health Data 2: e2. pp.5.

Alexander N.S., Wint W. (2013). Projected Population Proximity Indices (30km) for 2005, 2030 & 2050. Journal of Open Public Health Data 1: e2. http://dx.doi.org/10.5334/jophd.ab.

Allan B.F., Keesing F., Ostfeld R.S. (2003). Effect of forest fragmentation on Lyme disease risk. Conserv Biol 17: 267–272.

Alonso-Padilla J., Loza-Rubio E., Escribano-Romero E., Cordoba L., Cuevas S., Mejia F., Calderon R., Milian F., Travassos Da Rosa A., Weaver S.C., Estrada-Franco J.G., Saiz J.C. (2009). The continuous spread of West Nile virus (WNV): seroprevalence in asymptomatic horses. Epidemiol Infect 137: 1163-1168.

Amato-Gauci A., Zeller H. (2012). Tick-borne encephalitis joins the diseases under surveillance in the European Union. Euro Surveill 17.

ANB. (2015). Wildedierenziekten Surveillances. Agentschap Natuur en Bos Website. [In Dutch]. http://www.natuurenbos.be/beleid-wetgeving/overlast-schade/wildedierenziekten/surveillances/everzwijn-bewaking-op-ziekte-van.

Andreassen A., Jore S., Cuber P., Dudman S., Tengs T., Isaksen K., Hygen H.O., Viljugrein H., Anestad G., Ottesen P., Vainio K. (2012). Prevalence of tick borne encephalitis virus in tick nymphs in relation to climatic factors on the southern coast of Norway. Parasit Vectors 5: 177.

Andzhaparidze O.G., Rozina E.E., Bogomolova N.N., Boriskin Y.S. (1978). Morphological characteristics of the infection of animals with tick-borne encephalitis virus persisting for a long time in cell cultures. Acta Virol 22: 218-224.

Anonymous. (2004). OIE Manual of Standards for Diagnostic Tests and Vaccines. List A and B Diseases of Mammals, Birds and Bees, 3rd edition. Paris: 244-257.

Apollonio M., Andersen R., Putman R. (2010). European ungulates and their management in the 21st century. Cambridge University Press, Cambridge, United Kingdom.

Arino O., Ramos Perez J., Julio J., Kalogirou V., Bontemps S., Defourny P., van Bogaert E. (2012). Global Land Cover Map for 2009 (GlobCover 2009). ©European Space Agency (ESA) & Université catholique de Louvain (UCL).

Ashraf U., Ye J., Ruan X., Wan S., Zhu B., Cao S. (2015). Usutu virus: an emerging flavivirus in Europe. Viruses 7: 219-238.

Aslan Basbulut E., Gozalan A., Sonmez C., Coplu N., Korhasan B., Esen B., Akin L., Ertek M. (2012). [Seroprevalence of Borrelia burgdorferi and tick-borne encephalitis virus in a rural area of Samsun, Turkey]. Mikrobiyol Bul 46: 247-256.

Asmera J., Heinz F. (1972). Delimitation of natural foci of tick-borne encephalitis in north-eastern Moravia. Folia Parasitol (Praha) 19: 263-271.

Avia-GIS. (2012). EMMA Modelling Module in development for the VECMAP system. Produced by Avia-GIS Zoersel Belgium; ERGO Ltd. Oxford, UK; MEDES, Toulouse, France for the European Space Agency. http://www.european-mammals.org/php/mapmaker.php.

Baggott D., Ollagnier C., Yoon S.S., Collidor N., Mallouk Y., Cramer L.G. (2011). Efficacy of a novel combination of fipronil, amitraz and (S)-methoprene for treatment and control of tick species infesting dogs in Europe. Vet Parasitol 179: 330-334.

Bago Z., Bauder B., Kolodziejek J., Nowotny N., Weissenbock H. (2002). Tickborne encephalitis in a mouflon (Ovis ammon musimon). Vet Rec 150: 218-220.

Bajer A., Rodo A., Bednarska M., Mierzejewska E., Welc-Faleciak R. (2013). Babesia canis and tick-borne encephalitis virus (TBEV) co-infection in a sled dog. Ann Agric Environ Med 20: 426-430.

Bakhvalova V.N., Dobrotvorsky A.K., Panov V.V., Matveeva V.A., Tkachev S.E., Morozova O.V. (2006). Natural tick-borne encephalitis virus infection among wild small mammals in the southeastern part of western Siberia, Russia. Vector Borne Zoonotic Dis 6: 32-41.

Balling A., Plessow U., Beer M., Pfeffer M. (2014). Prevalence of antibodies against tick-borne encephalitis virus in wild game from Saxony, Germany. Ticks Tick Borne Dis.

Balmer D., Ruzek S., Ludwig S., Giardino A.P. (2007a). Learning about systems-based practice in the informal curriculum: a case study in an academic pediatric continuity clinic. Ambul Pediatr 7: 214-219.

Balmer D., Serwint J.R., Ruzek S.B., Ludwig S., Giardino A.P. (2007b). Learning behind the scenes: perceptions and observations of role modeling in pediatric residents' continuity experience. Ambul Pediatr 7: 176-181.

Balogh Z., Egyed L., Ferenczi E., Ban E., Szomor K.N., Takacs M., Berencsi G. (2012). Experimental infection of goats with tick-borne encephalitis virus and the possibilities to prevent virus transmission by raw goat milk. Intervirology 55: 194-200.

Balogh Z., Ferenczi E., Szeles K., Stefanoff P., Gut W., Szomor K.N., Takacs M., Berencsi G. (2010). Tick-borne encephalitis outbreak in Hungary due to consumption of raw goat milk. J Virol Methods 163: 481-485.

Banoo S., Bell D., Bossuyt P., Herring A., Mabey D., Poole F., Smith P.G., Sriram N., Wongsrichanalai C., Linke R., O'Brien R., Perkins M., Cunningham J., Matsoso P., Nathanson C.M., Olliaro P., Peeling R.W., Ramsay A. Evaluation of diagnostic tests for infectious diseases: general principles. Nat Rev Micro.

Barandika J.F., Hurtado A., Juste R.A., Garcia-Perez A.L. (2010). Seasonal dynamics of Ixodes ricinus in a 3-year period in northern Spain: first survey on the presence of tick-borne encephalitis virus. Vector Borne Zoonotic Dis 10: 1027-1035.

Barrett P., Plotkin S., Ehrlich H. (2008). Tick borne encephalitis virus vaccines. In: Vaccines (S. Plotkin, W. Orenstein & P. Offit, eds.).Saunders Elsevier. pp. 841.

Barrio I.C., Acevedo P., Tortosa F.S. (2010). Assessment of methods for estimating wild rabbit population abundance in agricultural landscapes. Eur J Wildl Res 56: 335-340.

Baumhackl U. (2009). Tick-borne encephalitis – TBE. In: Proceedings of the International Conference: Climate change impact on ticks and tick-borne diseases Belgium 2/2/2009.

Baxter. (2013). FSME-Verbreitungsgebiete in Europa. Stand: mai 2013 www.zecken.de.

BCFI-vet. (2014). Gecommentarieerd Geneesmiddelenrepertorium voor diergeneeskundig gebruik. Belgian Centre for Pharmacotherapeutic Information, Veterinary http://www.cbip-vet.be/nl/nlcomment.php - [Dutch or French].

BCFI. (2015). Ziekte van Lyme, een update. Folia Pharmaco-therapeutica, Maandelijks Tijdschrift Belgisch Centrum voor Farmacotherapeutische Informatie 42(5): 37-42.

Beck C., Jimenez-Clavero M.A., Leblond A., Durand B., Nowotny N., Leparc-Goffart I., Zientara S., Jourdain E., Lecollinet S. (2013). Flaviviruses in Europe: complex circulation patterns and their consequences for the diagnosis and control of West Nile disease. Int J Environ Res Public Health 10: 6049-6083.

Beck S., Schreiber C., Schein E., Krucken J., Baldermann C., Pachnicke S., von Samson-Himmelstjerna G., Kohn B. (2014). Tick infestation and prophylaxis of dogs in northeastern Germany: a prospective study. Ticks Tick Borne Dis 5: 336-342.

Belikov S.I., Kondratov I.G., Potapova U.V., Leonova G.N. (2014). The relationship between the structure of the tick-borne encephalitis virus strains and their pathogenic properties. PLoS One 9: e94946.

BELSPO-VGT10. Emerging diseases: more disease outbreaks in the future: pp. 4. http://eoedu.belspo.be/en/profs/vgt-europe-diseases.asp?section=1.3.4.

Beltrame A., Ruscio M., Cruciatti B., Londero A., Di Piazza V., Copetti R., Moretti V., Rossi P., Gigli G.L., Scudeller L., Viale P. (2006). Tickborne encephalitis virus, northeastern Italy. Emerg Infect Dis 12: 1617-1619.

Berrada Z.L., Telford S.R., 3rd. (2009). Burden of tick-borne infections on American companion animals. Top Companion Anim Med 24: 175-181.

Bertrand Y., Topel M., Elvang A., Melik W., Johansson M. (2012). First dating of a recombination event in mammalian tick-borne flaviviruses. PLoS One 7: e31981.

Beugnet F., Chalvet-Monfray K., Loukos H. (2009). FleaTickRisk: a meteorological model developed to monitor and predict the activity and density of three tick species and the cat flea in Europe. Geospat Health 4: 97-113.

Beugnet F., Marie J.L. (2009). Emerging arthropod-borne diseases of companion animals in Europe. Vet Parasitol 163: 298-305.

Biernat B., Cieniuch S., Stanczak J. (2014a). Detection of TBEV RNA in Ixodes ricinus ticks in north-eastern Poland. Ann Agric Environ Med 21: 689-692.

Biernat B., Karbowiak G. (2014). Study on the occurrence of tick-borne encephalitis virus RNA in European bison (Bison bonasus) eliminated at Bialowieza Primeval Forest (north-eastern Poland) in 2005-2009. Ann Parasitol 60: 99-102.

Biernat B., Karbowiak G., Werszko J., Stanczak J. (2014b). Prevalence of tick-borne encephalitis virus (TBEV) RNA in Dermacentor reticulatus ticks from natural and urban environment, Poland. Exp Appl Acarol.

Bingsohn L., Beckert A., Zehner R., Kuch U., Oehme R., Kraiczy P., Amendt J. (2013). Prevalences of tick-borne encephalitis virus and Borrelia burgdorferi sensu lato in Ixodes ricinus populations of the Rhine-Main region, Germany. Ticks Tick Borne Dis 4: 207-213.

Birkett M.A., Hassanali A., Hoglund S., Pettersson J., Pickett J.A. (2011). Repellent activity of catmint, Nepeta cataria, and iridoid nepetalactone isomers against Afro-tropical mosquitoes, ixodid ticks and red poultry mites. Phytochemistry 72: 109-114.

Bjöersdorff A. (2002). Borreliosis and tick-borne encephalitis. In: Proceedings of the International Conference: Climate change impact on ticks and tick-borne diseases Brussels, Belgium 2/2/2009. .

Bleyenheuft C., Lernout T., Berger N., Rebolledo J., Leroy M., Robert A., Quoilin S. (2015). Epidemiological situation of Lyme borreliosis in Belgium, 2003 to 2012. Arch Public Health 73: 015-0079.

Boadella M., Diez-Delgado I., Gutierrez-Guzman A.V., Hofle U., Gortazar C. (2012). Do wild ungulates allow improved monitoring of flavivirus circulation in Spain? Vector Borne Zoonotic Dis 12: 490-495.

Boadella M., Gortazar C., Acevedo P., Carta T., Martín-Hernando M.P., Fuente J., Vicente J. (2011). Six recommendations for improving monitoring of diseases shared with wildlife: examples regarding mycobacterial infections in Spain. European Journal of Wildlife Research 57: 697-706.

Bonenfant C., Gaillard J.-M. (2015). European roe deer, Capreolus capreolus. In: Acevedo P. and Putman R. (Eds.) Network for wildlife health surveillance in Europe Species Card. EWDA, APHAEA, EMIDA ERA-NET: pp. 6. http://www.aphaea.eu/sites/default/files/card_extern/aphaea_sc_roe_deer_060715.pdf.

Borcic B., Raos B., Kranzelic D., Abu Eldan J., Filipovic V. (1990). [The role of large wildlife in the maintenance of natural foci of tick-borne meningoencephalitis in northern Croatia]. Acta Med Iugosl 44: 399-406.

Bormane A., Lucenko I., Duks A., Mavtchoutko V., Ranka R., Salmina K., Baumanis V. (2004). Vectors of tick-borne diseases and epidemiological situation in Latvia in 1993-2002. Int J Med Microbiol 293 Suppl 37: 36-47.

Bosch J., Peris S., Fonseca C., Martinez M., De La Torre A., Iglesias I., Munoz M.J. (2012). Distribution, abundance and density of the wild boar on the Iberian Peninsula, based on the CORINE program and hunting statistics. Folia Zool 61: 138-151.

Bottieau E., Van Esbroeck M., Cnops L., Clerinx J., Van Gompel A. (2009). Chikungunya infection confirmed in a Belgian traveller returning from Phuket (Thailand). Euro Surveill 14.

Braks M., Medlock J.M., Hubalek Z., Hjertqvist M., Perrin Y., Lancelot R., Duchyene E., Hendrickx G., Stroo A., Heyman P., Sprong H. (2014). Vector-borne disease intelligence: strategies to deal with disease burden and threats. Front Public Health 2.

Brinkley C., Nolskog P., Golovljova I., Lundkvist A., Bergstrom T. (2008). Tick-borneencephalitis virus natural foci emerge in western Sweden. Int. J. Med. Microbiol. 298(44): 73–80.

Broker M. (2002). Tick-borne encephalitis virus within and outside Japan: a cause for concern? Jpn J Infect Dis 55: 55-56.

Brown L.D., Cai T.T., DasGupta A. (2001). Interval Estimation for a Binomial Proportion. Statistical Science 16(2): 101-133.

Brownstein J.S., Holford T.R., Fish D. (2003). A climate-based model predicts the spatial distribution of the Lyme disease vector Ixodes scapularis in the United States. Environ Health Perspect 111: 1152-1157.

Brownstein J.S., Skelly D.K., Holford T.R., Fish D. (2005). Forest fragmentation predicts local scale heterogeneity of Lyme disease risk. Oecologia 146: 469-475.

Brummer-Korvenkontio M., Saikku P., Korhonen P., Oker-Blom N. (1973). Arboviruses in Finnland. I. Isolation of Tick-Borne Encephalitis (TBE) Virus from Arthrophods, Vertebrates, and Patients. Amer. J. Trop. Med. Hyg. 22: 382–289.

Bryssinckx W., Ducheyne E., Leirs H., Hendrickx G. (2014). Optimizing denominator data estimation through a multimodel approach. Geospat Health 8: 573-582.

Bryssinckx W., Ducheyne E., Muhwezi B., Godfrey S., Mintiens K., Leirs H., Hendrickx G. (2012). Improving the accuracy of livestock distribution estimates through spatial interpolation. Geospat Health 7: 101-109.

BSAVA. (2009). PETS or pests? Facing the future of pet travel. News&Reports. The Veterinary Record 164: 446-448.

Buckland S.T., Anderson D.R., Burnham K.P., Laake J.L., Borchers D.L., Thomas L. (2004). Advanced Distance Sampling. Oxford University Press, Oxford. pp. 434.

Bunnell J.E., Price S.D., Das A., Shields T.M., Glass G.E. (2003). Geographic information systems and spatial analysis of adult Ixodes scapularis (Acari: Ixodidae) in the Middle Atlantic region of the U.S.A. J Med Entomol 40: 570-576.

Burri C., Bastic V., Maeder G., Patalas E., Gern L. (2011). Microclimate and the zoonotic cycle of tick-borne encephalitis virus in Switzerland. J Med Entomol 48: 615-627.

Burri C., Korva M., Bastic V., Knap N., Avsic-Zupanc T., Gern L. (2012). Serological evidence of tick-borne encephalitis virus infection in rodents captured at four sites in Switzerland. J Med Entomol 49: 436-439.

Caini S., Szomor K., Ferenczi E., Szekelyne Gaspar A., Csohan A., Krisztalovics K., Molnar Z., Horvath J. (2012). Tick-borne encephalitis transmitted by unpasteurised cow milk in western Hungary, September to October 2011. Euro Surveill 17.

Callens S. (2016). Encephalitis clinical approach of uncommon, emerging and traveling causes of encephalitis (UZ Gent - AZ Sint. Lucas). [in Dutch]. Seminar: diagnosis and surveillance of infectious diseases WIV-ISP - Spring symposium BVIKM/SBIMC

19/05/2016. https://epidemio.wiv-isp.be/ID/Pages/Ssid_32_presentations.aspx.

Cameron A. (1999). Survey Toolbox for Livestock Diseases. A practical manual and software package for active surveillance in developing countries. ACIAR Monograph N°54, vii: pp.330 http://epitools.ausvet.com.au/content.php?page=SurveyToolbox

Cargnelutti B., Spitz F., Valet G. (1992). Analysis of dispersion of wild boar (Sus scrofa) in Southern France. . In: Spitz F., Janeau G., Gonzales G., Aulagnier S. (eds) Proceedings of the 'Ongulés/Ungulates 91' International Symposium. Société Francaise pour l'Etude et la Protection des Mammifères, Paris, and Institut de Recherche sur les Grands Mammifères, Toulouse, France.: 423-425.

Carpi G., Bertolotti L., Rosati S., Rizzoli A. (2009). Prevalence and genetic variability of tick-borne encephalitis virus in host-seeking Ixodes ricinus in northern Italy. J Gen Virol 90: 2877-2883.

Carpi G., Cagnacci F., Neteler M., Rizzoli A. (2008). Tick infestation on roe deer in relation to geographic and remotely sensed climatic variables in a tick-borne encephalitis endemic area. Epidemiol Infect 136: 1416-1424.

Casaer J., Licoppe A. (2010). Ungulates and their management in Belgium. In: Apollonio M., Andersen R., Putman R. (eds.) European ungulates and their management in the 21st century. Cambridge University Press, New York, pp. 184-200.

CDC. (2014a). Preventing Ticks in the Yard: Apply Pesticides Outdoors to Control Ticks. http://www.cdc.gov/ticks/avoid/in_the_yard.html.

CDC. (2014b). Tick-borne Encephalitis (TBE) Fact Sheet. http://www.cdc.gov/vhf/tbe/index.html.

CDC. (2015). Rabies-Free Countries and Political Units. CDC Website. http://www.cdc.gov/importation/rabies-free-countries.html.

Cerny V. (1975). The role of mammals in natural foci of tick-borne encephalitis in Central Europe. Folia Parasitol (Praha) 22: 271-273.

CFSPH. (2009). Louping Ill. Animal Disease Information Technical Fact Sheet The Center for Food Security and Public Health – Iowa State University: pp. 4. http://www.cfsph.iastate.edu/Factsheets/pdfs/louping_ill.pdf.

CFSPH. (2013). West Nile Virus Infection. Animal Disease Information Technical Fact Sheet. The Center for Food Security and Public Health – Iowa State University: pp. 19. http://www.cfsph.iastate.edu/Factsheets/pdfs/west_nile_fever.pdf.

Chambers T.J., Diamond M.S. (2003). Pathogenesis of flavivirus encephalitis. Adv Virus Res 60: 273-342.

Charrel R.N., Attoui H., Butenko A.M., Clegg J.C., Deubel V., Frolova T.V., Gould E.A., Gritsun T.S., Heinz F.X., Labuda M., Lashkevich V.A., Loktev V., Lundkvist A., Lvov D.V., Mandl C.W., Niedrig M., Papa A., Petrov V.S., Plyusnin A., Randolph S., Suss J., Zlobin V.I., de Lamballerie X. (2004). Tick-borne virus diseases of human interest in Europe. Clin Microbiol Infect 10: 1040-1055.

Chausov E.V., Ternovoi V.A., Protopopova E.V., Kononova J.V., Konovalova S.N., Pershikova N.L., Romanenko V.N., Ivanova N.V., Bolshakova N.P., Moskvitina N.S., Loktev V.B. (2010). Variability of the tick-borne encephalitis virus genome in the 5' noncoding region derived from ticks Ixodes persulcatus and Ixodes pavlovskyi in Western Siberia. Vector Borne Zoonotic Dis 10: 365-375.

Chiba N., Iwasaki T., Mizutani T., Kariwa H., Kurata T., Takashima I. (1999). Pathogenicity of tick-borne encephalitis virus isolated in Hokkaido, Japan in mouse model. Vaccine 17: 779-787.

Chomel B. (2013). Chapter 8: Synthesis and zoonotic aspects. In: Beugnet, F. (Ed.) Guide to vector borne diseases of pets. Merial, Lyon, France. ISBN: 978-2-915758-40-5. : 399-416.

Cisak E., Wójcik-Fatla A., Sroka J., Zając V., Bilska-Zając E., Chmurzyńska E., Dutkiewicz J. (2012). Prevalence of Tick-Borne Encephalitis Virus Antibodies in Domestic and Game Animals from Eastern Poland. Bulletin of the Veterinary Institute in Pulawy 56: 275–278.

Cisak E., Wojcik-Fatla A., Zajac V., Sroka J., Buczek A., Dutkiewicz J. (2010). Prevalence of tick-borne encephalitis virus (TBEV) in samples of raw milk taken randomly from cows, goats and sheep in eastern Poland. Ann Agric Environ Med 17: 283-286.

Claerebout E., Losson B., Cochez C., Casaert S., Dalemans A.C., De Cat A., Madder M., Saegerman C., Heyman P., Lempereur L. (2013). Ticks and associated pathogens collected from dogs and cats in Belgium. Parasit Vectors 6: 183.

Clarke D.H., Casals J. (1958). Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Am J Trop Med Hyg 7: 561-573.

CLC. (2006). Corine Land Cover seamless vector GIS data. Green urban areas. http://www.eea.europa.eu/data-and-maps/data/clc-2006-vector-data-version-2.

Clement J., Van Ranst M. (2002). Lyme epidemiology in Belgium. 18th Symposium (spring 2002) on Tick-borne diseases in Belgium: http://www.sbimc.org/2002/spring/Clement/sld2002.htm.

Conover M.R., Vail R.M. (2015). 13.6 What people can do to reduce their risk of contracting Lyme disease. In: Human Diseases from Wildlife. CRC Press - Taylor and Francis Group, London. ISBN 978-1-4665-6215-8: 201-202.

CoreTeamR. (2013). A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.

Csángó P.A., Blakstad E., Kirtz G.C., Pedersen J.E., Czettel B. (2004). Tick-borne encephalitis in southern Norway. Emerg Infect Dis 10: 533-534.

Cuber P., Andreassen A., Vainio K., Asman M., Dudman S., Szilman P., Szilman E., Ottesen P., Anestad G., Ciesla-Nobis S., Solarz K. (2015). Risk of exposure to ticks (Ixodidae) and the prevalence of tick-borne encephalitis virus (TBEV) in ticks in Southern Poland. Ticks Tick Borne Dis 6: 356-363.

CWHC. (2015). Wildlife Submission Forms. Canadian Wildlife Health Cooperative Report and Submit. http://www.cwhc-rcsf.ca/report_submit.php.

Daniel M., Benes C., Danielova V., Kriz B. (2011). Sixty years of research of tick-borne encephalitis--a basis of the current knowledge of the epidemiological situation in Central Europe. Epidemiol Mikrobiol Imunol 60: 135-155.

Daniel M., Kolar J., Zeman P., Pavelka K., Sadlo J. (1998). Predictive map of Ixodes ricinus high-incidence habitats and a tick-borne encephalitis risk assessment using satellite data. Exp Appl Acarol 22: 417-433.

Dantas-Torres F., Chomel B.B., Otranto D. (2012). Ticks and tick-borne diseases: a One Health perspective. Trends Parasitol 28: 437-446.

Das A., Lele S.R., Glass G.E., Shields T., Patz J. (2002). Modeling a discrete spatial response using generalized linear mixed models: application to lyme disease vectors. International Journal of Geographical Information Science 16(2): 151-166.

De Bosschere H., Saegerman C., Neukermans A., Berkvens D., Casaer J., Vanopdenbosch E., Roels S. (2006). First chronic wasting disease (CWD) surveillance of roe deer (Capreolus capreolus) in the northern part of Belgium. Vet Q 28: 55-60.

De Craeye S. (2012). Toxoplasma gondii, a successful and underestimated foodborne parasite: development of detection methods and their use for the screening of animal reservoirs. Dissertation (monograph) in Veterinary Sciences. Department of Virology, parasitology and immunology, UGent, Belgium. ISBN: 9789058642820: pp. 188.

De Keukeleire M., Vanwambeke S.O., Somasse E., Kabamba B., Luyasu V., Robert A. (2015). Scouts, forests, and ticks: Impact of landscapes on human-tick contacts. Ticks Tick Borne Dis 27: 00100-00104.

de la Fuente J., Kocan K.M. (2003). Advances in the identification and characterization of protective antigens for recombinant vaccines against tick infestations. Expert Rev Vaccines 2: 583-593.

de la Fuente J., Moreno-Cid J.A., Canales M., Villar M., de la Lastra J.M., Kocan K.M., Galindo R.C., Almazan C., Blouin E.F. (2011). Targeting arthropod subolesin/akirin for the development of a universal vaccine for control of vector infestations and pathogen transmission. Vet Parasitol 181: 17-22.

Debiasi R.L., Tyler K.L. (2004). Molecular methods for diagnosis of viral encephalitis. Clin Microbiol Rev 17: 903-925, table of contents.

Deblinger R.D., Rimmer D.W. (1991). Efficacy of a permethrin-based acaricide to reduce the abundance of Ixodes dammini (Acari: Ixodidae). J Med Entomol 28: 708-711.

DeLong E.R., DeLong D.M., Clarke-Pearson D.L. (1988). Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. Biometrics 44: 837-845.

Denk H., Kovac W. (1966). Die experimentelle Frühsommer-Encephalitis (FSEM) der weißen Maus. Acta Neuropathol (Berl) 7: 162–174.

DevelopmentTeam. (2014). Quantum GIS Geographic Information System. Valmiera 2.2.0.

. Open Source Geospatial Foundation Project http://www.qgis.org. http://docs.qgis.org/2.2/pdf/en/.

Devleesschauwer B., Torgerson P., Charlier J., Levecke B., Praet N., Dorny P., Berkvens D., Speybroeck N. (2013). Prevalence: Tools for prevalence assessment studies. . R package version 0.1.0. http://cran.r-project.org/package=prevalence.

DGARNE. (2015a). Cerf élaphe (Cervus elaphus) - Distribution. La biodiversité en Wallonie. http://biodiversite.wallonie.be/fr/cervus-elaphus.html?IDD=50333811&highlighttext=cerf+%C3%A9laphe+&IDC=324.

DGARNE. (2015b). Renard (Vulpes vulpes) - Distribution. La biodiversité en Wallonie. http://biodiversite.wallonie.be/fr/vulpes-vulpes.html?IDD=50333779&IDC=326.

DGARNE. (2015c). Sanglier (Sus scrofa) - Distribution La biodiversité de la Wallonie. http://biodiversite.wallonie.be/fr/sus-scrofa.html?IDD=50333810&IDC=324.
Dietz O., Huskamp B. (2005). Infektionskrankheiten des Zentralnervensystems: Frühsommer-Meningoenzephalitis (FSME). In: Handbuch Pferdepraxis Georg Thieme Verlag: p. 677.
Dister S.W., Fish D., Bros S.M., Frank D.H., Wood B.L. (1997). Landscape characterization of peridomestic risk for Lyme disease using satellite imagery. Am J Trop Med Hyg 57: 687-692.
Dobler G. (2010). Zoonotic tick-borne flaviviruses. Vet Microbiol 140: 221-228.
Dobler G., Essbauer S., Terzioglu R., Thomas A., Wolfel R. (2008). [Prevalence of tick-borne encephalitis virus and rickettsiae in ticks of the district Burgenland, Austria]. Wien Klin Wochenschr 120: 45-48.
Dobler G., Hufert F., Pfeffer M., Essbauer S. (2011). Tick-borne encephalitis: from microfocus to human disease. progress in parasitology. Parasitol. Res. Monogr. 2: 323–331.
Dohoo I., Martin W., Stryhn H. (2009a). Chapter 2: Sampling. Veterinary Epidemiologic Research, second edition. AVC Inc, Charlottetown, Prince Edward Island, Canada: 33-56.
Dohoo I., Martin W., Stryhn H. (2009b). Chapter 5: Screening and Diagnostic Tests. Veterinary Epidemiologic Research, second edition. AVC Inc, Charlottetown, Prince Edward Island, Canada: p. 91-134.
Dohoo I., Martin W., Stryhn H. (2009c). Chapter 11: Controlled studies. Veterinary Epidemiologic Research, second edition. AVC Inc, Charlottetown, Prince Edward Island, Canada: 229.
Dohoo I., Martin W., Stryhn H. (2009d). Chapter 16: Logistic Regression. Veterinary Epidemiologic Research, second edition. AVC Inc, Charlottetown, Prince Edward Island, Canada: 421.
Dohoo I., Martin W., Stryhn H. (2009e). Chapter 18: Modelling count and rate data. Veterinary Epidemiologic Research, second edition. AVC Inc, Charlottetown, Prince Edward Island, Canada: 462-464.
Dolan M.C., Maupin G.O., Schneider B.S., Denatale C., Hamon N., Cole C., Zeidner N.S., Stafford K.C., 3rd. (2004). Control of immature Ixodes scapularis (Acari: Ixodidae) on rodent reservoirs of Borrelia burgdorferi in a residential community of southeastern Connecticut. J Med Entomol 41: 1043-1054.
Domingo C., Escadafal C., Rumer L., Mendez J.A., Garcia P., Sall A.A., Teichmann A., Donoso-Mantke O., Niedrig M. (2012). First international external quality assessment study on molecular and serological methods for yellow fever diagnosis. PLoS One 7: 3.
Donoso Mantke O., Aberle S.W., Avsic-Zupanc T., Labuda M., Niedrig M. (2007a). Quality control assessment for the PCR diagnosis of tick-borne encephalitis virus infections. J Clin Virol 38: 73-77.
Donoso Mantke O., Achazi K., Niedrig M. (2007b). Serological Versus PCR Methods for the Detection of Tick-borne Encephalitis Virus Infections in Humans. Future Virology 2: 565-572.
Donoso Mantke O., Schadler R., Niedrig M. (2008a). A survey on cases of tick-borne encephalitis in European countries. Euro Surveill 13: 18848.
Donoso Mantke O., Vaheri A., Ambrose H., Koopmans M., de Ory F., Zeller H., Beyrer K., Windorfer A., Niedrig M. (2008b). Analysis of the surveillance situation for viral encephalitis and meningitis in Europe. Euro Surveill 13.
Drăgănescu N., Iftimovici R., Iacobescu V., Girjabu E., Buşilă A., Cvaşniuc D., Tudor G., Lapuşneanu C., Mănăstireanu M. (1975). Investigations on the presence of antibodies to several flaviviruses in humans and some domestic animals in a biotope with a high frequency of migratory birds. Virologie 26: 103-108.
Drelich A., Andreassen A., Vainio K., Kruszynski P., Wasik T.J. (2014). Prevalence of tick-borne encephalitis virus in a highly urbanized and low risk area in Southern Poland. Ticks Tick Borne Dis 5: 663-667.

Drewes S., Schmidt S., Jacob J., Imholt C., Ulrich R.G. (2015). Common vole, Microtus arvalis - Bank vole, Myodes glareolus - Yellow-necked mouse, Apodemus flavicollis - Wood mouse, Apodemus sylvaticus - Water vole, Arvicola amphibius - Southern water vole, Arvicola sapidus. In: Henttonen H. (Ed.) Network for wildlife health surveillance in Europe Species Card - EWDA, APHAEA, EMIDA ERA-NET: pp. 10. http://www.aphaea.eu/sites/default/files/card_extern/aphaea_sc_voles_mouses_28082015.pdf .

Dryden M.W. (2009). Flea and tick control in the 21st century: challenges and opportunities. Vet Dermatol 20: 435-440.

Ducoffre G. (2008a). Borrelia burgdorferi ; Anaplasma phagocytophilum. Surveillance van Infectieuze Aandoeningen door een Netwerk van Laboratoria voor microbiologie 2007; Epidemiologische Trends 1983 – 2006 Rapport D/2008/2505/21.

Ducoffre G. (2008b). Borrelia burgdorferi ; Anaplasma phagocytophilum. Surveillance van Infectieuze Aandoeningen door een Netwerk van Laboratoria voor microbiologie 2007 Epidemiologische Trends 1983 – 2006. Rapport D/2008/2505/21.

Dumpis U., Crook D., Oksi J. (1999). Tick-borne encephalitis. Clin Infect Dis 28: 882-890.

Duscher G.G., Leschnik M., Fuehrer H.P., Joachim A. (2014). Wildlife reservoirs for vector-borne canine, feline and zoonotic infections in Austria. Int J Parasitol Parasites Wildl 4: 88-96.

Duscher G.G., Leschnik M., Fuehrer H.P., Joachim A. (2015a). Wildlife reservoirs for vector-borne canine, feline and zoonotic infections in Austria. Int J Parasitol Parasites Wildl 4: 88-96.

Duscher G.G., Wetscher M., Baumgartner R., Walder G. (2015b). Roe deer sera used for TBE surveillance in Austria. Ticks Tick Borne Dis 6: 489-493.

Dzieciolowski R.M., Clarke C.M.H., Fredric B.J. (1990). Growth of feral pigs in New Zealand. Acta Theriologica 35 77-88.

EC. (2012). Commission Implementing Decision of 8 August 2012 amending Decision 2002/253/EC laying down case definitions for reporting communicable diseases to the Community network under Decision No 2119/98/EC of the European Parliament and of the Council (notified under document C(2012) 5538). Official Journal of the European Union L 262: p.34-35.

ECDC.2012. Epidemiological situation of tick-borne encephalitis in the European Union and European Free Trade Association countries (Stockholm, European Centre for Disease Prevention and Control).

ECDC. (2014). Tick-borne encephalitis. In: European Centre for Disease Prevention and Control. Annual epidemiological report 2014 – emerging and vector-borne diseases. Stockholm: ECDC - 2014. p 20-23.

ECDC, EFSA, VBORNET, VECTORNET. (2015). Ixodes ricinus - current known distribution - July 2015. http://ecdc.europa.eu/en/healthtopics/vectors/vector-maps/Pages/VBORNET-maps-tick-species.aspx.

EDENext. (2012). Distance weighted population proximity index v0.3 for 2005,2030 and 2050,for rural, urban and total populations. Spatial metadata standard derived from ISO 19115: 2003(E). 2012-04-30. METADATASTANDARDVERSION:1. http://www.edenextdata.com/?q=data.

EEA. (2012). Vector-borne diseases (CLIM 037) Assessment published Nov 2012, European Environment Agency, Copenhagen.: http://www.eea.europa.eu/data-and-maps/indicators/vector-bornediseases1/assessment.

Egyed L., Elo P., Sreter-Lancz Z., Szell Z., Balogh Z., Sreter T. (2012). Seasonal activity and tick-borne pathogen infection rates of Ixodes ricinus ticks in Hungary. Ticks Tick Borne Dis 3: 90-94.

Eichenberger R.M., Deplazes P., Mathis A. (2015). Ticks on dogs and cats: a pet owner-based survey in a rural town in northeastern Switzerland. Ticks Tick Borne Dis 6: 267-271.

Eisen L., Eisen R.J. (2011). Using geographic information systems and decision support systems for the prediction, prevention, and control of vector-borne diseases. Annu Rev Entomol 56: 41-61.
Engeman R.M., Massei G., Sage M., Gentle M.N. (2013). Monitoring wild pig populations: a review of methods. Environ Sci Pollut Res Int 20: 8077-8091.
ENIVD. (2014). Commercial diagnostic tests available. European Network for Diagnostics of Imported Viral Diseases.
Ensoy C., Faes C., Welby S., Van der Stede Y., Aerts M. (2014). Exploring cattle movements in Belgium. Prev Vet Med 116: 89-101.
Ernek E., Kozuch O. (1970). Zeckenencephalitis-Virus neutralisierende Antikörper bei Rindern als Indikator der Virusanwesenheit in mitteleuropäischen Naturherden. Zbl. Bakt. Parasit. 213.
Escribano-Romero E., Lupulovic D., Merino-Ramos T., Blazquez A.B., Lazic G., Lazic S., Saiz J.C., Petrovic T. (2015). West Nile virus serosurveillance in pigs, wild boars, and roe deer in Serbia. Vet Microbiol 176: 365-369.
Estrada-Pena A. (2002a). Increasing habitat suitability in the United States for the tick that transmits Lyme disease: a remote sensing approach. Environ Health Perspect 110: 635-640.
Estrada-Pena A. (2002b). Understanding the relationships between landscape connectivity and abundance of Ixodes ricinus ticks. Exp Appl Acarol 28: 239-248.
Estrada-Pena A., Ortega C., Sanchez N., Desimone L., Sudre B., Suk J.E., Semenza J.C. (2011). Correlation of Borrelia burgdorferi sensu lato prevalence in questing Ixodes ricinus ticks with specific abiotic traits in the western palearctic. Appl Environ Microbiol 77: 3838-3845.
Everaert D., Geysen D., Brandt J., Witters J. (2007). First reported case of bovine babesiosis in Flanders. Vlaams Diergeneeskd. Tijdschr. 76 208-215.
EWDA. (2015). Diagnosis Cards AIV, Rabies, TB, Trichinella, BTV, EHD. EWDA Website. https://sites.google.com/site/ewdawebsite/diagnosis-cards.
Fajs L., Durmisi E., Knap N., Strle F., Avsic-Zupanc T. (2012). Phylogeographic characterization of tick-borne encephalitis virus from patients, rodents and ticks in Slovenia. PLoS One 7: e48420.
Falco R.C., Fish D. (1992). A comparison of methods for sampling the deer tick, Ixodes dammini, in a Lyme disease endemic area. Exp Appl Acarol 14: 165-173.
Falco R.C., McKenna D.F., Daniels T.J., Nadelman R.B., Nowakowski J., Fish D., Wormser G.P. (1999). Temporal relation between Ixodes scapularis abundance and risk for Lyme disease associated with erythema migrans. Am J Epidemiol 149: 771-776.
Famerée L., Cotteleer C., Antoine H. (1977). La babésiose bovine en Belgique, une anthropozoonose envahissante et méconnue. Incidence des babésioses animales sur la santé humaine. Rev Med Liege 32: 383–390.
FAO. (2001). Global ecological zones of Europe. Global Ecological Zoning for the Global Forest Resources Assessment FRA 2000 Rome, 2001: 151-153.
FASFC. (2011). West Nile virus/Virus du Nil occidental Activiteitenverslag 2010/Rapport d'activités 2010. Federal Agency for the Safety of the Food Chain, Brussels, Belgium, 2011-07. [Dutch/French]. http://www.favv.be/rapportsannuels/_documents/2012-06-26_RA2011Fr_S.pdf.
FASFC. (2012). West Nile virus/Virus du Nil occidental Activiteitenverslag 2011/Rapport d'activités 2011. Federal Agency for the Safety of the Food Chain, Brussels, Belgium, 2012-06-26. [Dutch/French]. http://www.favv.be/rapportsannuels/_documents/2012-06-26_RA2011Fr_S.pdf.
FASFC. (2013). West Nile virus/Virus du Nil occidental Activiteitenverslag 2012/Rapport d'activités 2012. Federal Agency for the Safety of the Food Chain, Brussels, Belgium, 2013-07-23. [Dutch/French]. http://www.favv.be/rapportsannuels/_documents/2013-07-23_RA_2012FR.pdf.

FASFC. (2014a). Volet 1 - étude transversale / Luik 1: - cross-sectionele studie Vademecum: Organisation de la Campagne d'hiver 2015 / Vademecum Organisatie van de Winterscreening 2015 [in French or Dutch]. FASFC: Belgian Federal Agency for the Safety of the Food Chain. : p. 3. http://www.favv.be/santeanimale/_documents/2014_2011_2001_Vademecum_Organisationcampagnedhiver2015_fr.pdf or http://www.favv.be/dierengezondheid/_documents/2014_2011_2001_Vademecum_Organisatievandewinterscreening2015_nl.pdf.

FASFC. (2014b). West Nile virus/Virus du Nil occidental. Activiteitenverslag 2013/Rapport d'activités 2013. Federal Agency for the Safety of the Food Chain, Brussels, Belgium, 2014-07-09. [Dutch/French]. http://www.favv.be/rapportsannuels/_documents/2014-07-09_RA_2013_Fr.pdf.

FASFC. (2015a). West Nile koorts (of West Nile fever)/Fièvre du Nil occidental (ou fièvre de West Nile). . Belgian Federal Agency for the Safety of the Food Chain Website. [Dutch/French]. http://www.favv.be/dierengezondheid/westnilekoorts/ or http://www.favv.be/santeanimale/fievreniloccidental/

FASFC. (2015b). West Nile virus/Virus du Nil occidental. Activiteitenverslag 2014/Rapport d'activités 2014. Federal Agency for the Safety of the Food Chain, Brussels, Belgium, 15-07-2015. [Dutch/French]. http://www.afsca.be/activiteitenverslag/2014/gezondheid/dierengezondheid/#westnile.

FASFC. (2016). Sanitel. http://www.favv-afsca.fgov.be/dierlijkeproductie/dieren/sanitel/ - http://www.favv-afsca.fgov.be/productionanimale/animaux/sanitel/.

Fernández-de-Simón J., Díaz-Ruiz F., Cirilli F., Tortosa F.S., Villafuerte R., Delibes-Mateos M. (2011). Towards a standardized index of European rabbit abundance in Iberian Mediterranean habitats. Eur J Wildl Res 57: 1091-1100.

Fleiss J.L., Levin B., Paik M.C. (2003). Statistical Methods for Rates and Proportions, third edition. John Wiley & Sons, London 598-626.

FLI. (2015). Imported Animals (1979 - 2015).Rabies Information System of the WHO Collaboration Centre for Rabies Surveillance and Research. Rabies - Bulletin - Europe. http://www.who-rabies-bulletin.org/About_Rabies/Imported/Animals.aspx.

Foldvari G., Farkas R. (2005). Ixodid tick species attaching to dogs in Hungary. Vet Parasitol 129: 125-131.

Fomsgaard A., Christiansen C., Bodker R. (2009). First identification of tick-borne encephalitis in Denmark outside of Bornholm, August 2009. Euro Surveill 14.

Fomsgaard A., Fertner M.E., Essbauer S., Nielsen A.Y., Frey S., Lindblom P., Lindgren P.E., Bodker R., Weidmann M., Dobler G. (2013). Tick-borne encephalitis virus, Zealand, Denmark, 2011. Emerg Infect Dis 19: 1171-1173.

Formanova P., Cerny J., Bolfikova B.C., Valdes J.J., Kozlova I., Dzhioev Y., Ruzek D. (2015). Full genome sequences and molecular characterization of tick-borne encephalitis virus strains isolated from human patients. Ticks Tick Borne Dis 6: 38-46.

Frantzidou F., Kamaria F., Dumaidi K., Skoura L., Antoniadis A., Papa A. (2008). Aseptic meningitis and encephalitis because of herpesviruses and enteroviruses in an immunocompetent adult population. Eur J Neurol 15: 995-997.

Frimmel S., Krienke A., Riebold D., Lobermann M., Littmann M., Fiedler K., Klaus C., Suss J., Reisinger E.C. (2010). [Tick-borne encephalitis virus in humans and ticks in Northeastern Germany]. Dtsch Med Wochenschr 135: 1393-1396.

Frimmel S., Krienke A., Riebold D., Loebermann M., Littmann M., Fiedler K., Klaus C., Suss J., Reisinger E.C. (2014). Tick-borne encephalitis virus habitats in North East Germany: reemergence of TBEV in ticks after 15 years of inactivity. Biomed Res Int 2014: 308371.

Garigliany M.M., Marlier D., Tenner-Racz K., Eiden M., Cassart D., Gandar F., Beer M., Schmidt-Chanasit J., Desmecht D. (2014). Detection of Usutu virus in a bullfinch (Pyrrhula pyrrhula) and a great spotted woodpecker (Dendrocopos major) in north-west Europe. Vet J 199: 191-193.

Gaston W., Armstrong J.B., Arjo W., Stribling H.L. (2008). Home Range and Habitat Use of Feral Hogs (Sus scrofa) on Lowndes County WMA, Alabama. National Conference on Feral Hogs. Paper 6. http://digitalcommons.unl.edu/feralhog/6.

Gaumann R., Muhlemann K., Strasser M., Beuret C.M. (2010). High-throughput procedure for tick surveys of tick-borne encephalitis virus and its application in a national surveillance study in Switzerland. Appl Environ Microbiol 76: 4241-4249.

GBIF. (2015a). Capreolus capreulus Linnaeus, 1758. Species in GBIF Backbone Taxonomy 2013-07-01. Global Biodiverisity Information Facility Secretariat. Accessed on 2015-10-26 via http://www.gbif.org/species/5220126.

GBIF. (2015b). Sus scrofa Linnaeus, 1758. Species in GBIF Backbone Taxonomy 2013-07-01. Global Biodiverisity Information Facility Secretariat. Accessed on 2015-10-26 via http://www.gbif.org/species/2441218.

Gehrt S.D., Wilson E.C., Brown J.L., Anchor C. (2013). Population ecology of free-roaming cats and interference competition by coyotes in urban parks. PLoS One 8.

Geller J., Nazarova L., Katargina O., Leivits A., Jarvekulg L., Golovljova I. (2013). Tick-borne pathogens in ticks feeding on migratory passerines in Western part of estonia. Vector Borne Zoonotic Dis 13: 443-448.

George J.E. (1990). Summing-up of strategies for the control of ticks in regions of the world other than Africa. Parassitologia 32: 203-209.

Gerth H.J., Grimshandl D., Stage B., Doller G., Kunz C. (1995). Roe deer as sentinels for endemicity of tick-borne encephalitis virus. Epidemiol Infect 115: 355-365.

Girjabu E., Draganescu N., Iftimovici R. (1985). Serological investigations on the presence of antibodies to tick-borne encephalitis virus in domestic animals and birds and in humans. Virologie 36: 161-164.

Glaser C.A., Gilliam S., Schnurr D. (2003). In search of encephalitis etiologies: diagnostic challenges in the California Encephalitis Project, 1998-2000. Clin Infect Dis: 36:731.

Glass G.E., Schwartz B.S., Morgan J.M., 3rd, Johnson D.T., Noy P.M., Israel E. (1995). Environmental risk factors for Lyme disease identified with geographic information systems. Am J Public Health 85: 944-948.

Golovljova I., Vene S., Sjolander K.B., Vasilenko V., Plyusnin A., Lundkvist A. (2004). Characterization of tick-borne encephalitis virus from Estonia. J Med Virol 74: 580-588.

Gómez-Martínez C. (2014). Role of cervids and wild boar on the presence of tick-borne encephalitis virus in Sweden / Hjortdjurs och vildsvins roll för förekomsten av fästingburen encefalit i Sverige. Examensarbete i ämnet biologi, Department of Wildlife, Fish, and Environmental studies, Sveriges lantbruksuniversitet / Swedish University of Agricultural Sciences Umeå: pp. 17.

Gortazar C., Acevedo P., Ferroglio E. (2014a). Eurasian wild boar, Sus scrofa. Apollonio M. and Keuling O. (Eds.) Network for wildlife health surveillance in Europe Species Card. EWDA, APHAEA, EMIDA ERA-NET: pp. 5. http://www.aphaea.eu/cards-e/wildboar.

Gortazar C., Diez-Delgado I., Baransona J.A., Vicente J., De La Fuente J., Boadella M. (2015). The wild side of disease control at the wildlife-livestock-human interface: a review. Frontiers in Veterinary Science 1(27): pp.12.

Gortázar C., Höfle U., Esperon F. (2015). Wild birds, Aves. In: Casas F. and Newton I. (Eds.) Network for wildlife health surveillance in Europe Species Card: pp. 5. http://www.aphaea.eu/sites/default/files/card_extern/aphaea_sc_wildbirds_051115b.pdf.

Gortazar C., Reperant L.A., Kuiken T., de la Fuente J., Boadella M., Martinez-Lopez B., Ruiz-Fons F., Estrada-Pena A., Drosten C., Medley G., Ostfeld R., Peterson T., VerCauteren K.C., Menge C., Artois M., Schultsz C., Delahay R., Serra-Cobo J., Poulin R., Keck F., Aguirre A.A., Henttonen H., Dobson A.P., Kutz S., Lubroth J., Mysterud A. (2014b). Crossing the interspecies barrier: opening the door to zoonotic pathogens. PLoS Pathog 10: e1004129.

Gould E.A., Higgs S., Buckley A., Gritsun T.S. (2006). Potential arbovirus emergence and implications for the United Kingdom. Emerg Infect Dis 12: 549-555.

Gould E.A., Moss S.R., Turner S.L. (2004). Evolution and dispersal of encephalitic flaviviruses. Arch Virol Suppl: 65-84.

Grabner A. (1993). Klinische Differentialdiagnose infektiös bedingter Krankheiten des ZNS beim Pferd mit besonderer Berücksichtigung der EHV- Infektionen. Prakt. Tierarzt, Colleg. Vet. XXIV: 27-31.

Grard G., Moureau G., Charrel R.N., Lemasson J.J., Gonzalez J.P., Gallian P., Gritsun T.S., Holmes E.C., Gould E.A., de Lamballerie X. (2007). Genetic characterization of tick-borne flaviviruses: new insights into evolution, pathogenetic determinants and taxonomy. Virology 361: 80-92.

Grear J.S., Koethe R., Hoskins B., Hillger R., Dapsis L., Pongsiri M. (2014). The effectiveness of permethrin-treated deer stations for control of the Lyme disease vector Ixodes scapularis on Cape Cod and the islands: a five-year experiment. Parasit Vectors 7: 1756-3305.

Greene C.E. (2013). Chapter 24: Arthropod-borne Viral Infections. In: Greene (Ed.) Infectious Diseases of the Dog and Cat (4th Ed.). Elsevier, Saunders, USA.: p. 210-218.

Gregory R.D., Gibbons D.W., Donald P.F. (2004). Bird census and survey techniques. Chapter 2 In: Sutherland W.J., Newton I. ,Green R. (Eds.) "Bird Ecology and Conservation: A Handbook of Techniques", Oxford University Press, Oxford. ISBN 0 19 852085 9.

Gresikova M., Kaluzova M. (1997). Biology of tick-borne encephalitis virus. Acta Virol 41: 115-124.

Gresikova M., Noseck J. (1966). Isolation of tick-borne encephalitis virus from Haemaphysalis inermis ticks. Acta Virol 10: 359-361.

Gresikova M., Sekeyova M., Weidnerova K., Blaskovic D., Steck F., Wandeler A. (1972). Isolation of tick-borne encephalitis virus from the brain of a sick dog in Switzerland. Acta Virol 16: 88.

Grešíková M., Weidnerova K., Nosek J., Rajcani J. (1972). Experimental pathogenicity of tick-borne encephalitis virus for dogs. Acta Virol 16: 336-340.

Gritsun T.S., Lashkevich V.A., Gould E.A. (2003a). Tick-borne encephalitis. Antiviral Res 57: 129-146.

Gritsun T.S., Nuttall P.A., Gould E.A. (2003b). Tick-borne flaviviruses. Adv Virus Res 61: 317-371.

Gubler D.J., Kuno G., Markoff L. (2007). Flaviviruses. In: Knipe D.M., Griffin D.E., Lamb R.A., Straus S.E., Howley P.M., Martin M.A., Roizman B. (editors). Fields Virology, 5th edition, Philadelphia: Lippincott, Wolters, : 1200-1203.

Guerra M., Walker E., Jones C., Paskewitz S., Cortinas M.R., Stancil A., Beck L., Bobo M., Kitron U. (2002). Predicting the risk of Lyme disease: habitat suitability for Ixodes scapularis in the north central United States. Emerg Infect Dis 8: 289-297.

Guerrero F.D., Miller R.J., Perez de Leon A.A. (2012). Cattle tick vaccines: many candidate antigens, but will a commercially viable product emerge? Int J Parasitol 42: 421-427.

Günes T., Poyraz O., Atas M., Alim A. (2010). [Seroprevalence of tick-borne encephalitis virus (TBEV) among the residents of rural areas in Sinop, central Black-Sea region, Turkey]. Mikrobiyol Bul 44: 585-591.

Gunther G., Haglund M. (2005). Tick-borne encephalopathies : epidemiology, diagnosis, treatment and prevention. CNS Drugs 19: 1009-1032.

Gunther G., Haglund M., Lindquist L., Forsgren M., Skoldenberg B. (1997). Tick-bone encephalitis in Sweden in relation to aseptic meningo-encephalitis of other etiology: a prospective study of clinical course and outcome. J Neurol 244: 230-238.

Hadorn D.C., Stärk K.D. (2008). Evaluation and optimization of surveillance systems for rare and emerging infectious diseases. Vet Res 39: 25.

Haemig P.D., Sjostedt de Luna S., Grafstrom A., Lithner S., Lundkvist A., Waldenstrom J., Kindberg J., Stedt J., Olsen B. (2011). Forecasting risk of tick-borne encephalitis (TBE): using data from wildlife and climate to predict next year's number of human victims. Scand J Infect Dis 43: 366-372.

Haglund M. (2002). Occurrence of TBE in areas previously considered being non-endemic: Scandinavian data generate an international study by the International Scientific Working Group for TBE (ISW-TBE). Int J Med Microbiol 291 Suppl 33: 50-54.

Haglund M., Forsgren M., Lindh G., Lindquist L. (1996). A 10-year follow-up study of tick-borne encephalitis in the Stockholm area and a review of the literature: need for a vaccination strategy. Scand J Infect Dis 28: 217-224.

Haglund M., Günther G. (2003). Tick-borne encephalitis—pathogenesis, clinical course and long-term follow-up. Vaccine 21, Supplement 1: S11-S18.

Haglund M., Settergren B., Heinz F.X., Günther G. (2003). Report of the Meningitis Program of the International Scientific Working Group on TBE: Serological screening of patients with viral CNS-infection of unknown etiology in search of undiagnosed TBE cases. Vaccine 21, Supplement 1: S66-S72.

Han X., Aho M., Vene S., Peltomaa M., Vaheri A., Vapalahti O. (2001). Prevalence of tick-borne encephalitis virus in Ixodes ricinus ticks in Finland. J Med Virol 64: 21-28.

Han X., Juceviciene A., Uzcategui N.Y., Brummer-Korvenkontio H., Zygutiene M., Jaaskelainen A., Leinikki P., Vapalahti O. (2005). Molecular epidemiology of tick-borne encephalitis virus in Ixodes ricinus ticks in Lithuania. J Med Virol 77: 249-256.

Hanski I., Henttonen H., Hansson L. (1994). Temporal variability of population density in microtine rodents: a reply to Xia and Boonstra. American Naturalist 144: 329-342.

Harrell T., Hammes J.S. (2012). Meningitis admitted to a military hospital: a retrospective case series. Mil Med 177: 1223-1226.

Hartemink N., Vanwambeke S.O., Purse B.V., Gilbert M., Van Dyck H. (2014). Towards a resource-based habitat approach for spatial modelling of vector-borne disease risks. Biol Rev Camb Philos Soc 22: 12149.

Hayasaka D., Suzuki Y., Kariwa H., Ivanov L., Volkov V., Demenev V., Mizutani T., Gojobori T., Takashima I. (1999). Phylogenetic and virulence analysis of tick-borne encephalitis viruses from Japan and far-Eastern Russia. J Gen Virol 80 (Pt 12): 3127-3135.

Heinz F., Kunz C. (1975). Ein sensitives Gewebekultur-Antigen des Friihsommerenzephalitis-Viruszur Verwendung im Hamagglutinationshemmungstest. Arch Virol Arch Virol 48: 191-194.

Heinz F.X. (2003). Molecular aspects of TBE virus research. Vaccine 21, Supplement 1: S3-S10.

Heinz F.X. (2008). Etiology. In: Compendium of Tick-borne encephalitis (TBE, FSME). Monograph, Baxter, ISW-TBE Working Group, .

Heinz F.X., Allison S.L. (2000). Structures and mechanisms in flavivirus fusion. Adv Virus Res 55: 231-269.

Herpe B., Schuffenecker I., Pillot J., Malvy D., Clouzeau B., Bui N., Vargas F., Gruson D., Zeller H., Lafon M.E., Fleury H., Hilbert G. (2007). Tickborne encephalitis, southwestern France. Emerg Infect Dis 13: 1114-1116.

Heylen D., Adriaensen F., Van Dongen S., Sprong H., Matthysen E. (2013). Ecological factors that determine Ixodes ricinus tick burdens in the great tit (Parus major), an avian reservoir of Borrelia burgdorferi s.l. Int J Parasitol 43: 603-611.

Heylen D., Matthysen E., Fonville M., Sprong H. (2014). Songbirds as general transmitters but selective amplifiers of Borrelia burgdorferi sensu lato genotypes in Ixodes rinicus ticks. Environ Microbiol 16: 2859-2868.

Heyman P. (2009). Climate change impact on ticks and tick-borne diseases. Proceedings of the International Conference: Climate change impact on ticks and tick-borne diseases Brussels, Belgium 2/2/2009.

Higgins A.J., Snyder J.R. (2006). Chapter 1: Infectious diseases - Lyme disease (Borreliosis). In: The equine manual, Elsevier Saunders. ISBN: 978-0-7020-2769-7: 88-89.

Holbach M., Oehme R. (2002). [Tick-borne encephalitis and Lyme borreliosis. Spread of pathogens and risk of illness in a tick-borne encephalitis region]. Fortschr Med Orig 120: 113-118.

Holzmann H. (2003). Diagnosis of tick-borne encephalitis. Vaccine 21, Supplement 1: S36-S40.

Holzmann H., Aberle S.W., Stiasny K., Werner P., Mischak A., Zainer B., Netzer M., Koppi S., Bechter E., Heinz F.X. (2009). Tick-borne encephalitis from eating goat cheese in a mountain region of Austria. Emerg Infect Dis 15: 1671-1673.

Holzmann H., Kundi M., Stiasny K., Clement J., McKenna P., Kunz C., Heinz F.X. (1996). Correlation between ELISA, hemagglutination inhibition, and neutralization tests after vaccination against tick-borne encephalitis. J Med Virol 48: 102-107.

Horger M., Beck R., Fenchel M., Ernemann U., Nagele T., Brodoefel H., Heckl S. (2012). Imaging findings in tick-borne encephalitis with differential diagnostic considerations. AJR Am J Roentgenol 199: 420-427.

Horn J.A., Mateus-Pinilla N., Warner R.E., Heske E.J. (2011). Home range, habitat use, and activity patterns of free-roaming domestic cats. The Journal of Wildlife Management 75: 1177-1185.

Hoyaux J., Verheyden S., Bogaert J., Ghelen Y., van Leest M., Van der Avort A., Squilbin M. (2011). Chapter 3: Impacts of climate change in Belgium. National Adaptation Strategy Report. Edited by the National Climate Commission – December 2010. http://www.cnc-nkc.be: pp.12. http://www.lne.be/themas/klimaatverandering/adaptatie/bestandenmap/naschapter13.pdf/view.

Huang C., Slater B., Campbell W., Howard J., White D. (2001). Detection of arboviral RNA directly from mosquito homogenates by reverse-transcription-polymerase chain reaction. J Virol Methods 94: 121-128.

Hubalek Z., Cerny V., Mittermayer T., Kilik J., Halouzka J., Juricova Z., Kuhn I., Bardos V. (1986). Arbovirological survey in Silica plateau area, Roznava District, Czechoslovakia. J Hyg Epidemiol Microbiol Immunol 30: 87-98.

Hubalek Z., Juricova Z., Svobodova S., Halouzka J. (1993). A serologic survey for some bacterial and viral zoonoses in game animals in the Czech Republic. J Wildl Dis 29: 604-607.

Hubalek Z., Pow I., Reid H.W., Hussain M.H. (1995). Antigenic similarity of central European encephalitis and louping-ill viruses. Acta Virol 39: 251-256.

Hubalek Z., Rudolf I. (2012). Tick-borne viruses in Europe. Parasitol Res 111: 9-36.

Hubalek Z., Rudolf I., Nowotny N. (2014). Arboviruses pathogenic for domestic and wild animals. Adv Virus Res 89: 201-275.

Hudopisk N., Korva M., Janet E., Simetinger M., Grgic-Vitek M., Gubensek J., Natek V., Kraigher A., Strle F., Avsic-Zupanc T. (2013). Tick-borne encephalitis associated with consumption of raw goat milk, Slovenia, 2012. Emerg Infect Dis 19: 806-808.

Hudson P.J., Rizzoli A., Rosa R., Chemini C., Jones L.D., Gould E.A. (2001). Tick-borne encephalitis virus in northern Italy: molecular analysis, relationships with density and seasonal dynamics of Ixodes ricinus. Med Vet Entomol 15: 304-313.

Hunink M., Glasziou P. (2001). Decision Making in Health and Medicine - Integrating Evidence and Values. Cambridge University Press. 128-156.

Ikawa-Yoshida A., Yoshii K., Kuwahara K., Obara M., Kariwa H., Takashima I. (2011). Development of an ELISA system for tick-borne encephalitis virus infection in rodents. Microbiol Immunol 55: 100-107.

Imhoff M., Hagedorn P., Schulze Y., Hellenbrand W., Pfeffer M., Niedrig M. (2015). Review: Sentinels of tick-borne encephalitis risk. Ticks Tick Borne Dis 6: 592-600.

Imperio S., Ferrante M., Grignetti A., Santini G., Focardi S. (2010). Investigating population dynamics in ungulates: do hunting statistics make up a good index of population abundance? Wildlife Biol 16.

ISW-TBE, Baxter. (2006). Epidemiology 2006 Tick-Borne Encephalitis (TBE, FSME) and Epidemiological map 2006. pp. 12. http://www.tbe-info.com/upload/medialibrary/Reported_Cases.pdf.

ISW-TBE, Baxter. (2009). Map TBE/FSME* in Europe 2008. International Scientific Working Group on Tick-Borne Encephalitis Copyright Baxter – Hölzel Verlag. http://www.isw-tbe.info/upload/medialibrary/FSME_inet_klein_e2009.jpg

ISW-TBE, Baxter. (2011a). Map TBE/FSME* in Europe 2010. International Scientific Working Group on Tick-Borne Encephalitis and Baxter – Hölzel Verlag, Vienna, Austria. http://www.isw-tbe.info/upload/medialibrary/FSME_inet_klein_e2011.jpg.

ISW-TBE, Baxter. (2011b). Map TBE/FSME* in Europe 2011. International Scientific Working Group on Tick-Borne Encephalitis and Baxter – Hölzel Verlag, Vienna, Austria. http://www.tbe-info.com/upload/medialibrary/EndemicAreas2011.jpg.

ISW-TBE, Baxter. (2013). Map TBE/FSME* in Europe 2012. International Scientific Working Group on Tick-Borne Encephalitis and Baxter – Hölzel Verlag, Vienna, Austria. http://www.isw-tbe.info/upload/medialibrary/FSME_inet_klein_e2013.jpg

ITG.2008. Precautions against ticks, Lyme disease and TBE/FSME (Antwerp, Belgium, Prince Leopold Institute of Tropical Medicine).

IUCN. (2012). The IUCN Red List of Threatened Species, Version 2012.2. Available at: http://www.iucnredlist.

Jääskeläinen A.E., Sironen T., Murueva G.B., Subbotina N., Alekseev A.N., Castren J., Alitalo I., Vaheri A., Vapalahti O. (2010). Tick-borne encephalitis virus in ticks in Finland, Russian Karelia and Buryatia. J Gen Virol 91: 2706-2712.

Jääskeläinen A.E., Tikkakoski T., Uzcategui N.Y., Alekseev A.N., Vaheri A., Vapalahti O. (2006). Siberian subtype tickborne encephalitis virus, Finland. Emerg Infect Dis 12: 1568-1571.

Jaenson T.G., Eisen L., Comstedt P., Mejlon H.A., Lindgren E., Bergstrom S., Olsen B. (2009). Risk indicators for the tick Ixodes ricinus and Borrelia burgdorferi sensu lato in Sweden. Med Vet Entomol 23: 226-237.

Jaenson T.G., Garboui S., Palsson K. (2006). Repellency of oils of lemon eucalyptus, geranium, and lavender and the mosquito repellent MyggA natural to Ixodes ricinus (Acari: Ixodidae) in the laboratory and field. J Med Entomol 43: 731-736.

Jaenson T.G., Hjertqvist M., Bergstrom T., Lundkvist A. (2012). Why is tick-borne encephalitis increasing? A review of the key factors causing the increasing incidence of human TBE in Sweden. Parasit Vectors 5: 184.

Jaenson T.G., Talleklint L., Lundqvist L., Olsen B., Chirico J., Mejlon H. (1994). Geographical distribution, host associations, and vector roles of ticks (Acari: Ixodidae, Argasidae) in Sweden. J Med Entomol 31: 240-256.

Jahfari S., de Vries A., Rijks J., Van Gucht S., Sprong H., Rockx B. ([submitted]). Tick-borne encephalitis virus found in the Netherlands, 2015. Euro Surveill.

James C. (2008). Fästingburen encefalit (TBE) hos hund. Bla Stjärnans Djursjukhus, Göteborg: 1-20. [in Swedish].

James M.C., Bowman A.S., Forbes K.J., Lewis F., McLeod J.E., Gilbert L. (2013). Environmental determinants of Ixodes ricinus ticks and the incidence of Borrelia burgdorferi sensu lato, the agent of Lyme borreliosis, in Scotland. Parasitology 140: 237-246.

Janitza-Futterer D. (2003). Serologische Untersuchungen zur endemischen Situation der Infektion mit dem FSME-Virus in einer südbadischen Pferde- und Hundepopulation. Dissertation, LMU München: Faculty of Veterinary Medicine: pp. 171.

Jarrin I., Sellier P., Lopes A., Morgand M., Makovec T., Delcey V., Champion K., Simoneau G., Green A., Mouly S., Bergmann J.F., Lloret-Linares C. (2016). Etiologies and Management of Aseptic Meningitis in Patients Admitted to an Internal Medicine Department. Medicine 95: 0000000000002372.

Jemersic L., Dezdek D., Brnic D., Prpic J., Janicki Z., Keros T., Roic B., Slavica A., Terzic S., Konjevic D., Beck R. (2014). Detection and genetic characterization of tick-borne encephalitis virus (TBEV) derived from ticks removed from red foxes (Vulpes vulpes) and isolated from spleen samples of red deer (Cervus elaphus) in Croatia. Ticks Tick Borne Dis 5: 7-13.

Jimenez-Clavero M.A., Tejedor C.G., Rojo G., Soriguer R., Figuerola J. (2007). Serosurvey of West Nile virus in equids and bovids in Spain. Vet Rec 161: 212.

Jongejan F., Fourie J.J., Chester S.T., Manavella C., Mallouk Y., Pollmeier M.G., Baggott D. (2011). The prevention of transmission of Babesia canis canis by Dermacentor reticulatus ticks to dogs using a novel combination of fipronil, amitraz and (S)-methoprene. Vet Parasitol 179: 343-350.

Jongejan F., Ringenier M., Putting M., Berger L., Burgers S., Kortekaas R., Lenssen J., van Roessel M., Wijnveld M., Madder M. (2015). Novel foci of Dermacentor reticulatus ticks infected with Babesia canis and Babesia caballi in the Netherlands and in Belgium. Parasit Vectors 8: 232.

Jongejan F., Uilenberg G. (2013). Chapter 2.1 Panorama of vector borne diseases of pets in Europe In: Beugnet, F. (Ed.) Guide to vector borne diseases of pets. Merial, Lyon, France. ISBN: 978-2-915758-40-5. : pp. 425.

Jonsson N.N., Matschoss A.L., Pepper P., Green P.E., Albrecht M.S., Hungerford J., Ansell J. (2000). Evaluation of tickGARD(PLUS), a novel vaccine against Boophilus microplus, in lactating Holstein-Friesian cows. Vet Parasitol 88: 275-285.

Juceviciene A., Zygutiene M., Leinikki P., Brummer-Korvenkontio H., Salminen M., Han X., Vapalahti O. (2005). Tick-borne encephalitis virus infections in Lithuanian domestic animals and ticks. Scand J Infect Dis 37: 742-746.

Juricova Z. (1992). [Arbovirus antibodies in wild game caught in Moravia]. Vet Med (Praha) 37: 633-636.

Juricova Z., Hubalek Z. (1999). Serological surveys for arboviruses in the game animals of southern Moravia (Czech Republic). Folia Zool. 48(3): 185-189.

Kaiser R. (1999). The clinical and epidemiological profile of tick-borne encephalitis in southern Germany 1994-98: a prospective study of 656 patients. Brain 122 (Pt 11): 2067-2078.

Kaiser R. (2008a). Clinical Description. In: Compendium of Tick-borne encephalitis (TBE, FSME) Monograph, Baxter, ISW-TBE: 21-28.

Kaiser R. (2008b). Tick-borne encephalitis. Infect Dis Clin North Am 22: 561-575, x.

Kallio-Kokko H., Uzcategui N., Vapalahti O., Vaheri A. (2005). Viral zoonoses in Europe. FEMS Microbiol Rev. 29: 1051-1077.

Kalluri S., Gilruth P., Rogers D., Szczur M. (2007). Surveillance of arthropod vector-borne infectious diseases using remote sensing techniques: a review. PLoS Pathog 3: 1361-1371.

Kaluzova M., Eleckova E., Zuffova E., Pastorek J., Kaluz S., Kozuch O., Labuda M. (1994). Reverted virulence of attenuated tick-borne encephalitis virus mutant is not accompanied with the changes in deduced viral envelope protein amino acid sequence. Acta Virol 38: 133-140.

Karbowiak G., Kiewra D. (2010). New locations of Dermacentor reticulatus ticks in Western Poland: the first evidence of the merge in D. reticulatus occurrence areas? Wiad Parazytol 56: 333-336.

Katargina O., Russakova S., Geller J., Kondrusik M., Zajkowska J., Zygutiene M., Bormane A., Trofimova J., Golovljova I. (2013). Detection and characterization of tick-borne encephalitis virus in Baltic countries and eastern Poland. PLoS One 8: e61374.

Kazarina A., Japina K., Keiss O., Salmane I., Bandere D., Capligina V., Ranka R. (2015). Detection of tick-borne encephalitis virus in I. ricinus ticks collected from autumn migratory birds in Latvia. Ticks Tick Borne Dis 6: 178-180.

Kerbo N., Donchenko I., Kutsar K., Vasilenko V. (2005). Tickborne encephalitis outbreak in Estonia linked to raw goat milk, May-June 2005. Euro Surveill 10: E050623 050622.

KICM. (2016). TICK-BORNE ENCEPHALITIS - SLOVAKIA: (KOSICE). . Infectology and Travel Medicine Department (KICM) of the Louis Pasteur University Hospital (UNLP): http://www.promedmail.org

Kießling J.R. (2005). Untersuchung zum Vorkommen des Frühsommer-Meningo-Enzephalitis-Virus und Borrelia burgdorferi in ausgewählten Wildmaus-und Zeckenpopulationen Bayerns. Inaugural-Dissertation Zur Erlangung der tiermedizinischen Doktorwürde der Tierärztlichen Fakultät der Ludwig-Maximilians-Universität München: pp.154.

Kiffner C., Lodige C., Alings M., Vor T., Ruhe F. (2011). Attachment site selection of ticks on roe deer, Capreolus capreolus. Exp Appl Acarol 53: 79-94.

Kiffner C., Vor T., Hagedorn P., Niedrig M., Ruhe F. (2012). Determinants of tick-borne encephalitis virus antibody presence in roe deer (Capreolus capreolus) sera. Med Vet Entomol 26: 18-25.

Kiffner C., Zucchini W., Schomaker P., Vor T., Hagedorn P., Niedrig M., Ruhe F. (2010). Determinants of tick-borne encephalitis in counties of southern Germany, 2001-2008. Int J Health Geogr 9: 42.

Kim S.Y., Yun S.M., Han M.G., Lee I.Y., Lee N.Y., Jeong Y.E., Lee B.C., Ju Y.R. (2008). Isolation of tick-borne encephalitis viruses from wild rodents, South Korea. Vector Borne Zoonotic Dis 8: 7-13.

Kipp S., , Goedecke A., Dorn W., Wilske B., Fingerle V. (2006). Role of birds in Thuringia, Germany, in the natural cycle of Borrelia burgdorferi sensu lato, the Lyme disease spirochaete. International Journal of Medical Microbiology 296 125-128.

Kirtz G. (1999). FSME-Infektion in einer ôsterreichischen Hundepopulation Diss. med. vet. Univ. Wien. : 151-160.

Kirtz G., Leschnik M., Leidinger E. (2001). Ixodes ricinus: Gemeingefährlich für Hundes! . Kleintierpraxis 46: 151-160. [in German].

Kitts-Morgan S.E., Caires K.C., Bohannon L.A., Parsons E.I., Hilburn K.A. (2015). Free-ranging farm cats: home range size and predation on a livestock unit in Northwest Georgia. PLoS One 10: e0120513.

Klaus C., Beer M., Saier R., Schau U., Moog U., Hoffmann B., Diller R., Suss J. (2012). Goats and sheep as sentinels for tick-borne encephalitis (TBE) virus--epidemiological studies in areas endemic and non-endemic for TBE virus in Germany. Ticks Tick Borne Dis 3: 27-37.

Klaus C., Beer M., Saier R., Schubert H., Bischoff S., Suss J. (2011). Evaluation of serological tests for detecting tick-borne encephalitis virus (TBEV) antibodies in animals. Berl Munch Tierarztl Wochenschr 124: 443-449.

Klaus C., Hoffmann B., Beer M., Muller W., Stark B., Bader W., Stiasny K., Heinz F.X., Suss J. (2010a). Seroprevalence of tick-borne encephalitis (TBE) in naturally exposed monkeys (Macaca sylvanus) and sheep and prevalence of TBE virus in ticks in a TBE endemic area in Germany. Ticks Tick Borne Dis 1: 141-144.

Klaus C., Hoffmann B., Hering U., Mielke B., Sachse K., Beer M., Suss J. (2010b). Tick-borne encephalitis (TBE) virus prevalence and virus genome characterization in field-collected ticks (Ixodes ricinus) from risk, non-risk and former risk areas of TBE, and in ticks removed from humans in Germany. Clin Microbiol Infect 16: 238-244.

Klaus C., Hoffmann B., Moog U., Schau U., Beer M., Suss J. (2010c). Can goats be used as sentinels for tick-borne encephalitis (TBE) in nonendemic areas? Experimental studies and epizootiological observations. Berl Munch Tierarztl Wochenschr 123: 441-445.

Klaus C., Horugel U., Hoffmann B., Beer M. (2013). Tick-borne encephalitis virus (TBEV) infection in horses: clinical and laboratory findings and epidemiological investigations. Vet Microbiol 163: 368-372.

Klaus C., Ziegler U., Kalthoff D., Hoffmann B., Beer M. (2014). Tick-borne encephalitis virus (TBEV) - findings on cross reactivity and longevity of TBEV antibodies in animal sera. BMC Vet Res 10: 78.

Klimeš J., Juricova Z., Literak I., Schanilec P., Trachta e Silva E. (2001). Prevalence of antibodies to tickborne encephalitis and West Nile flaviviruses and the clinical signs of tickborne encephalitis in dogs in the Czech Republic. Vet Rec 148: 17-20.

KMI. (2009). Klimatogrammen (België, Frankrijk, Duitsland). . Koninklijk Meteorologisch Instituut België.

Knap N., Avsic-Zupanc T. (2013). Correlation of TBE incidence with red deer and roe deer abundance in Slovenia. PLoS One 8.

Knap N., Korva M., Dolinsek V., Sekirnik M., Trilar T., Avsic-Zupanc T. (2012). Patterns of tick-borne encephalitis virus infection in rodents in Slovenia. Vector Borne Zoonotic Dis 12: 236-242.

Kollaritsch H., Chmelik V., Dontsenko I., Grzeszczuk A., Kondrusik M., Usonis V., Lakos A. (2011a). The current perspective on tick-borne encephalitis awareness and prevention in six Central and Eastern European countries: report from a meeting of experts convened to discuss TBE in their region. Vaccine 29: 4556-4564.

Kollaritsch H., Krasilnikov V., Holzmann H., Karganova G., Barrett A., Suss J., Pervikov Y., Bjorvatn B., Duclos P., Hombach J. (2011b). WHO Background Document on Vaccines and Vaccination against Tick-borne Encephalitis (TBE)*. . http://www.who.int/immunization/sage/6_TBE_backgr_18_Mar_net_apr_2011.pdf.

Korenberg E.I. (1994). Comparative ecology and epidemiology of lyme disease and tick-borne encephalitis in the former Soviet Union. Parasitol Today 10: 157-160.

Korenberg E.I., Kovalevskii Y.V. (1999). Main features of tick-borne encephalitis eco-epidemiology in Russia. Zentralbl Bakteriol 289: 525-539.

Korenberg E.I., Pchelkina A.A., Spitsina L.N. (1984). Consistent patterns in the contact of domestic animals with tick-borne encephalitis virus in the eastern part of the Russian plain. J Hyg Epidemiol Microbiol Immunol 28: 73-84.

Körting H. (1981). Problems of diagnosis and epidemiology of TBE. In: Kunz C, ed. Tick-borne encephalitis Wien: Facultas: 247-250.

Kozuch O., Labuda M., Lysy J., Weismann P., Krippel E. (1990). Longitudinal study of natural foci of Central European encephalitis virus in West Slovakia. Acta Virol 34: 537-544.

Kreil T.R., Zimmermann K., Burger I., Attakpah E., Mannhalter J.W., Eibl M.M. (1997). Detection of tick-borne encephalitis virus by sample transfer, plaque assay and strand-specific reverse transcriptase polymerase chain reaction: what do we detect? J Virol Methods 68: 1-8.

Kristiansen K. (2002). TBE in Denmark--in particular on Bornholm. Int J Med Microbiol 291 Suppl 33: 62-63.

Kriz B., Benes C., Daniel M. (2009). Alimentary transmission of tick-borne encephalitis in the Czech Republic (1997-2008). Epidemiol Mikrobiol Imunol 58: 98-103.

Kriz B., Daniel M., Benes C., Maly M. (2014). The role of game (wild boar and roe deer) in the spread of tick-borne encephalitis in the czech republic. Vector Borne Zoonotic Dis 14: 801-807.

Kunz C. (1992). Tick-borne encephalitis in Europe. Acta Leiden 60: 1-14.

Kunz C. (2003). TBE vaccination and the Austrian experience. Vaccine 21, Supplement 1: S50-S55.

Kunz C. (2008). Introduction. In: Compendium of Tick-borne encephalitis (TBE, FSME), Monograph, Baxter, ISW-TBE: 4.

Kunze M. (2008). Therapy. In: Compendium of Tick-borne encephalitis (TBE, FSME) Monograph, Baxter, ISW-TBE,: 29.

Kunze U. (2011). Tick-borne encephalitis: new paradigms in a changing vaccination environment. Wien Med Wochenschr 161: 361-364.

Kunze U. (2015). Tick-borne encephalitis as a notifiable disease--Status quo and the way forward. Report of the 17th annual meeting of the International Scientific Working Group on Tick-Borne Encephalitis (ISW-TBE). Ticks Tick Borne Dis 6: 545-548.

Kupca A.M., Essbauer S., Zoeller G., de Mendonca P.G., Brey R., Rinder M., Pfister K., Spiegel M., Doerrbecker B., Pfeffer M., Dobler G. (2010). Isolation and molecular characterization of a tick-borne encephalitis virus strain from a new tick-borne encephalitis focus with severe cases in Bavaria, Germany. Ticks Tick Borne Dis 1: 44-51.

Labuda M., Austyn J.M., Zuffova E., Kozuch O., Fuchsberger N., Lysy J., Nuttall P.A. (1996). Importance of localized skin infection in tick-borne encephalitis virus transmission. Virology 219: 357-366.

Labuda M., Danielova V., Jones L.D., Nuttall P.A. (1993a). Amplification of tick-borne encephalitis virus infection during co-feeding of ticks. Med Vet Entomol 7: 339-342.

Labuda M., Eleckova E., Lickova M., Sabo A. (2002). Tick-borne encephalitis virus foci in Slovakia. Int J Med Microbiol 33: 43-47.

Labuda M., Jones L.D., Williams T., Danielova V., Nuttall P.A. (1993b). Efficient transmission of tick-borne encephalitis virus between cofeeding ticks. J Med Entomol 30: 295-299.

Labuda M., Jones L.D., Williams T., Nuttall P.A. (1993c). Enhancement of tick-borne encephalitis virus transmission by tick salivary gland extracts. Med Vet Entomol 7: 193-196.

Labuda M., Kozuch, Lys E. (1997a). Tick-borne encephalitis virus natural foci in Slovakia: ticks, rodents, and goats. In: Tick-borne Encephalitis and Lyme Borreliosis, Potsdam Symposia (J. Suess and O. Kahl, eds.), Pabst Science Publishers, Lengerich: 34-46.

Labuda M., Kozuch O., Zuffova E., Eleckova E., Hails R.S., Nuttall P.A. (1997b). Tick-borne encephalitis virus transmission between ticks cofeeding on specific immune natural rodent hosts. Virology 235: 138-143.

Labuda M., Nuttall P.A., Kozuch O., Eleckova E., Williams T., Zuffova E., Sabo A. (1993d). Non-viraemic transmission of tick-borne encephalitis virus: a mechanism for arbovirus survival in nature. Experientia 49: 802-805.

Labuda M., Randolph S.E. (1999). Survival strategy of tick-borne encephalitis virus: cellular basis and environmental determinants. Zentralbl Bakteriol 289: 513-524.

Labuda M., Stunzner D., Kozuch O., Sixl W., Kocianova E., Schaffler R., Vyrostekova V. (1993e). Tick-borne encephalitis virus activity in Styria, Austria. Acta Virol 37: 187-190.

Labuda M., Trimnell A.R., Lickova M., Kazimirova M., Davies G.M., Lissina O., Hails R.S., Nuttall P.A. (2006). An antivector vaccine protects against a lethal vector-borne pathogen. PLoS Pathog 2: 7.

Lamarque F., Artois M. (1997). Surveillance of wildlife diseases in France: the SAGIR network. Epidemiol santé anim: 31-32.

Lambin E.F., Tran A., Vanwambeke S.O., Linard C., Soti V. (2010). Pathogenic landscapes: interactions between land, people, disease vectors, and their animal hosts. Int J Health Geogr 9: 9-54.

Landis J.R., Koch G.G. (1977). The measurement of observer agreement for categorical data. Biometrics 33: 159-174.

Laursen K., Knudsen J.D. (2003). Tick-borne encephalitis: a retrospective study of clinical cases in Bornholm, Denmark. Scand J Infect Dis 35: 354-357.

Leach M., Scoones I. (2013). The social and political lives of zoonotic disease models: narratives, science and policy. Soc Sci Med 88: 10-17.

Lean I.J., Rabiee A.R., Duffield T.F., Dohoo I.R. (2009). Invited review: Use of meta-analysis in animal health and reproduction: methods and applications. J Dairy Sci 92: 3545-3565.

Ledermann J.P., Lorono-Pino M.A., Ellis C., Saxton-Shaw K.D., Blitvich B.J., Beaty B.J., Bowen R.A., Powers A.M. (2011). Evaluation of widely used diagnostic tests to detect West Nile virus infections in horses previously infected with St. Louis encephalitis virus or dengue virus type 2. Clin Vaccine Immunol 18: 580-587.

Leeflang M.M., Bossuyt P.M., Irwig L. (2009). Diagnostic test accuracy may vary with prevalence: implications for evidence-based diagnosis. J Clin Epidemiol 62: 5-12.

Lempereur L., De Cat A., Caron Y., Madder M., Claerebout E., Saegerman C., Losson B. (2011). First molecular evidence of potentially zoonotic Babesia microti and Babesia sp. EU1 in Ixodes ricinus ticks in Belgium. Vector Borne Zoonotic Dis 11: 125-130.

Lempereur L., Lebrun M., Cuvelier P., Sepult G., Caron Y., Saegerman C., Shiels B., Losson B. (2012). Longitudinal field study on bovine Babesia spp. and Anaplasma phagocytophilum infections during a grazing season in Belgium. Parasitol Res 110: 1525-1530.

Lengerich E. (2016). 1.2 - Epidemiologic Triad - Lesson A Introduction to Epidemiology. In: STAT 507: Epidemiological Research Methods. Learning Online. https://onlinecourses.science.psu.edu/stat507.

Leonova G.N., Belikov S.I., Kondratov I.G., Takashima I. (2013). Comprehensive assessment of the genetics and virulence of tick-borne encephalitis virus strains isolated from patients with inapparent and clinical forms of the infection in the Russian Far East. Virology 443: 89-98.

Leprince D.J., Lane R.S. (1996). Evaluation of permethrin-impregnated cotton balls as potential nesting material to control ectoparasites of woodrats in California. J Med Entomol 33: 355-360.

Lernout T. (2016). Surveillance strategies for Lyme borreliosis in Belgium. Seminar Diagnosis and Surveillance of infectious diseases WIV-ISP - Spring symposium BVIKM/SBIMC - 19/05/2016. https://epidemio.wiv-isp.be/ID/Documents/Seminar/SSID_2016/Surveillance_strategies_for_Lyme_borreliosis%20_in_Belgium.pdf.

Leschnik M., Feiler A., Duscher G.G., Joachim A. (2013). Effect of owner-controlled acaricidal treatment on tick infestation and immune response to tick-borne pathogens in naturally infested dogs from Eastern Austria. Parasit Vectors 6: 1756-3305.

Leschnik M.W., Kirtz G.C., Thalhammer J.G. (2002). Tick-borne encephalitis (TBE) in dogs. Int J Med Microbiol 291 Suppl 33: 66-69.

Lešničar G., Poljak M., Seme K., Lešničar J. (1997). Pediatric tick-borne encephalitis in 371 cases from an endemic region in Slovenia, 1959 to 2000. Pediatric Infectious Disease Journal 22: 612-617.

Leutloff R., Nubling M., Neumann-Haefelin D., Rieger M., . . (2006). Cows as indicators for TBE endemic regions: suitability of testing for antibodies in serum and milk. Int J Med Microbiol 296(S40): 87–88.

Li S., Hartemink N., Speybroeck N., Vanwambeke S.O. (2012a). Consequences of landscape fragmentation on Lyme disease risk: a cellular automata approach. PLoS One 7: e39612.

Li S., Heyman P., Cochez C., Simons L., Vanwambeke S.O. (2012b). A multi-level analysis of the relationship between environmental factors and questing Ixodes ricinus dynamics in Belgium. Parasit Vectors 5: 149.

Libois R. (2006). L'érosion de la biodiversité : les mammifères. Partim «Les mammifères non volants». Dossier scientifique réalisé dans le cadre de l'élaboration du Rapport analytique 2006-2007 sur l'état de l'environnement wallon. Université de Liège. [In French]. pp. 127.

Linard C., Lamarque P., Heyman P., Ducoffre G., Luyasu V., Tersago K., Vanwambeke S.O., Lambin E.F. (2007). Determinants of the geographic distribution of Puumala virus and Lyme borreliosis infections in Belgium. Int J Health Geogr 6: 15.

Linard C., Vanwambeke S.O. (2009). Spatial distribution of TBE and Lyme borreliosis: landscape and beyond. Proceedings of the International Conference: Climate change impact on ticks and tick-borne diseases Brussels, Belgium 2/2/2009.

Lindblad G. (1960). A case of tick-borne encephalitis in a dog. Medlemsblad för Sveriges veterinärforbund 12: 416-417. [in Swedish].

Lindblom P., Wilhelmsson P., Fryland L., Sjowall J., Haglund M., Matussek A., Ernerudh J., Vene S., Nyman D., Andreassen A., Forsberg P., Lindgren P.E. (2014). Tick-borne encephalitis virus in ticks detached from humans and follow-up of serological and clinical response. Ticks Tick Borne Dis 5: 21-28.

Linden A. (2005). Epidémiosurveillance des maladies de la faune sauvage en Région Wallonne. [In French]. pp. 9. http://www.faunesauvage.be/faune-sauvage/.

Linden A., Gregoire F., Nahayo A., Hanrez D., Mousset B., Massart A.L., De Leeuw I., Vandemeulebroucke E., Vandenbussche F., De Clercq K. (2010). Bluetongue virus in wild deer, Belgium, 2005-2008. Emerg Infect Dis 16: 833-836.

Linden A., Mousset B., Gregoire F., Hanrez D., Vandenbussche F., Vandemeulebroucke E., Vanbinst T., Verheyden B., de Clerck K. (2008). Bluetongue virus antibodies in wild red deer in southern Belgium. Vet Rec. 2008 Apr 5;162(14):459.

Linden A., Wirtgen M., Nahayo A., Heyman P., Niedrig M., Schulze Y. (2012). Tickborne encephalitis virus antibodies in wild cervids in Belgium. Vet Rec 170: 108.

Lindgren E. (1998). Climate and tickborne encephalitis. . Conservation Ecology [online] 2(1): 5. http://www.consecol.org/vol2/iss1/art5/.

Lindgren E., Gustafson R. (2001). Tick-borne encephalitis in Sweden and climate change. Lancet 358: 16-18.

Lindh E., Huovilainen A., Ratti O., Ek-Kommonen C., Sironen T., Huhtamo E., Poysa H., Vaheri A., Vapalahti O. (2008). Orthomyxo-, paramyxo- and flavivirus infections in wild waterfowl in Finland. Virol J 5: 35.

Lindhe K.E., Meldgaard D.S., Jensen P.M., Houser G.A., Berendt M. (2009). Prevalence of tick-borne encephalitis virus antibodies in dogs from Denmark. Acta Vet Scand 51: 56.

Litzba N., Zelena H., Kreil T.R., Niklasson B., Kuhlmann-Rabens I., Remoli M.E., Niedrig M. (2014). Evaluation of different serological diagnostic methods for tick-borne encephalitis virus: enzyme-linked immunosorbent, immunofluorescence, and neutralization assay. Vector Borne Zoonotic Dis 14: 149-159.

Liverpool. (2015). UK Ticks. Tick Activity Project. University of Liverpool. Institute of Infection and Global health. https://www.liv.ac.uk/infection-and-global-health/research/zoonotic-infections/tick-activity-project/uk-ticks/.

Lizroth A., Quoilin S. (2009). Vereisten inzake belangrijke capaciteiten voor bewaking en bestrijding van noodsituaties van internationaal belang op het gebied van de volksgezondheid. Rapport d'activités. Project gefinancierd door DG2 – FOD VVVL. http://www.iph.fgov.be.

Lommano E., Burri C., Maeder G., Guerne M., Bastic V., Patalas E., Gern L. (2012). Prevalence and genotyping of tick-borne encephalitis virus in questing Ixodes ricinus ticks in a new endemic area in western Switzerland. J Med Entomol 49: 156-164.

Lommano E., Dvorak C., Vallotton L., Jenni L., Gern L. (2014). Tick-borne pathogens in ticks collected from breeding and migratory birds in Switzerland. Ticks Tick Borne Dis 5: 871-882.

Long M.T. (2011). Overview of Meningitis, Encephalitis, and Encephalomyelitis. The Merck Veterinary Manual Online http://www.merckmanuals.com/vet/nervous_system/meningitis_encephalitis_and_encephalomyelitis/overview_of_meningitis_encephalitis_and_encephalomyelitis.html?qt=tick%20borne%20encephalitis&alt=sh.

Losson B. (1989). Babesiose bovine en Belgique: a propos d'une enquête auprès des vétérinaires. Ann. Med. Vet. 133 63–67.

Losson B., Mollet J.J., Avez F., Malaise F., Mignon B. (1999). Description de trois cas autochtones de babesiose canine (Babesia canis) en Belgique. Annales de Médecine Vétérinaire 143: 119-124.

Luckschander N. (1998). FSME-Infektion in der Österreichischen Pferdepopulation. Dissertation aus der Wiener Veterinärmedizinischen Universität I. Medizinische TK.

Luckschander N., Kolbl S., Enzesberger O., Zipko H.T., Thalhammer J.G. (1999). Tick borne encephalitis (TBE) in an Austrian horse population. Tierarztl Prax Ausgabe Grostiere, Nutztiere 27(4): 235-238.

Luyasu V. (2009). How to protect the tourist: preventive measures for Lyme borreliosis and vaccine against tick-borne encephalitis. In: Proceedings of the International Conference: Climate change impact on ticks and tick-borne diseases Brussels, Belgium 2/2/2009.

Makowka A., Gut W., Rogalska J., Michalik J., Wodecka B., Rymaszewska A., Stefanoff P. (2009). [Detection of TBEV RNA in ticks as a tool for valuation of endemic area wide and sensitivity of TBE surveillance]. Przegl Epidemiol 63: 375-378.

Mandl C.W. (2005). Steps of the tick-borne encephalitis virus replication cycle that affect neuropathogenesis. Virus Res 111: 161-174.

Mansfield K.L., Balseiro Morales A., Johnson N., Ayllon N., Hofle U., Alberdi P., Fernandez de Mera I.G., Marin J.F., Gortazar C., de la Fuente J., Fooks A.R. (2015). Identification and characterization of a novel tick-borne flavivirus sub-type in goats (Capra hircus) in Spain. J Gen Virol 20: 000096.

Mansfield K.L., Johnson N., Phipps L.P., Stephenson J.R., Fooks A.R., Solomon T. (2009). Tick-borne encephalitis virus - a review of an emerging zoonosis. J Gen Virol 90: 1781-1794.

Markina F.A., Saez-Royuela C., De Garnica R. (2004). Physical development of wild boar in the Cantabric mountains, Alava, Northern Spain. Galemys 16: 25-34.

Marsot M., Henry P.-Y., Vourc'h G., Gasqui P., Ferquel E., Laignel J., Grysan M., Chapuis J.-L. (2012). Which forest bird species are the main hosts of the tick, Ixodes ricinus, the vector of Borrelia burgdorferi sensu lato, during the breeding season? International Journal for Parasitology 42.

Martínez M., Muñoz M.J., de la Torre A., Iglesias I., Peris S., Infante O., Sánchez-, Vizcaíno J.M. (2009). Risk of introduction of H5N1 HPAI from Europe to Spain by wild water birds in autumn. Transboundary and emerging diseases 56 86-98.

Matile H., Ferrari E., Aeschlimann A., Wyler R. (1981). [The transmission of tick-borne encephalitis in Switzerland. An attempt at establishing a register of natural reservoirs for a seroepidemiologic examination of forest personnel in the middle of the country]. Schweiz Med Wochenschr 111: 1262-1269.

Mazlo M., Szanto J. (1978). Morphological demonstration of the virus of tick-borne encephalitis in the human brain. Acta Neuropathol 43: 251-253.

MEDI. (2015). Chronic Wasting Disease - Wildlife issues. Michigan Emerging Disease Issues. Diseases that may affect humans or animals. http://www.michigan.gov/emergingdiseases/0,4579,7-186-25806_26401-135830--,00.html.

Mejlon H.A., Jaenson T.G., Mather T.N. (1995). Evaluation of host-targeted applications of permethrin for control of Borrelia-infected Ixodes ricinus (Acari: Ixodidae). Med Vet Entomol 9: 207-210.

Mercelis S. (2003). Edelhert - Cervus elaphus Linnaeus, 1758. . In: Verkem, S., De Maeseneer, J., Vandendriessche, B., Verbeylen, G. & Yskout, S. (Eds.) Zoogdieren in Vlaanderen. Ecologie en verspreiding van 1987 tot 2002. Natuurpunt Studie en JNM-Zoogdierenwerkgroep, Mechelen en Gent, België. [In Dutch]. http://waarnemingen.be/soort/info/388.

Merino F.J., Serrano J.L., Saz J.V., Nebreda T., Gegundez M., Beltran M. (2000). Epidemiological characteristics of dogs with Lyme borreliosis in the province of Soria (Spain). Eur J Epidemiol 16: 97-100.

Merino O., Alberdi P., Perez de la Lastra J.M., de la Fuente J. (2013). Tick vaccines and the control of tick-borne pathogens. Front Cell Infect Microbiol 3.

Meyer-Kayser E., Hoffmann L., Silaghi C., Pfister K., Mahling M., Passos L.M. (2012). Dynamics of tick infestations in foxes in Thuringia, Germany. Ticks Tick Borne Dis 3: 232-239.

Mickiene A. (2008). Diagnosis. In: Compendium of Tick-borne encephalitis (TBE, FSME) Monograph, Baxter, ISW-TBE: 29-31.

Mickiene A., Laiskonis A., Gunther G., Vene S., Lundkvist A., Lindquist L. (2002). Tickborne encephalitis in an area of high endemicity in lithuania: disease severity and long-term prognosis. Clin Infect Dis 35: 650-658.

Mikryukova T.P., Moskvitina N.S., Kononova Y.V., Korobitsyn I.G., Kartashov M.Y., Tyuten Kov O.Y., Protopopova E.V., Romanenko V.N., Chausov E.V., Gashkov S.I., Konovalova S.N., Moskvitin S.S., Tupota N.L., Sementsova A.O., Ternovoi V.A., Loktev V.B. (2014). Surveillance of tick-borne encephalitis virus in wild birds and ticks in Tomsk city and its suburbs (Western Siberia). Ticks Tick Borne Dis 5: 145-151.

Misonne M.C., Van Impe G., Hoet P.P. (1998). Genetic heterogeneity of Borrelia burgdorferi sensu lato in Ixodes ricinus ticks collected in Belgium. J Clin Microbiol 36: 3352-3354.

Morelle K., Podgórski T., Prévot C., Keuling O., Lehaire F., Lejeune P. (2015). Towards understanding wild boar Sus scrofa movement: a synthetic movement ecology approach. Mammal Review 45: 15-29.

Movila A., Alekseev A.N., Dubinina H.V., Toderas I. (2013). Detection of tick-borne pathogens in ticks from migratory birds in the Baltic region of Russia. Med Vet Entomol 27: 113-117.

Müller K., Konig M., Thiel H.J. (2006). Tick-borne encephalitis (TBE) with special emphasis on infection in horses. Dtsch Tierarztl Wochenschr 113: 147-151. [in German].

Müller W. (1997). TBE in the dog - seroepidemiological studies. In: (J. Süss, O. Kahl, eds.) Tick-borne Encephalitis and Lyme Borreliosis Pabst Science Publishers, Lengerich. : 204-218. http://www.alomed.de/sp_fsme-impfempfehlung.htm.

Müller W. (2000). FSME Seroprävalenz beim Hund in Deutschland. Abstract at the 9th InnLab Conference in Munich on 6.-8 April 2000 http://www.alomed.de/DI_innlab.htm - [in German].

Müller W. (2010). FSME Seroprävalenz beim Hund in Deutschland. Abstract at the 9th InnLab conference in Munich on 6.-8 April 2000 [in German]. Accessed on: http://www.alomed.de/DI_innlab.htm.

Munoz P.M., Boadella M., Arnal M., de Miguel M.J., Revilla M., Martinez D., Vicente J., Acevedo P., Oleaga A., Ruiz-Fons F., Marin C.M., Prieto J.M., de la Fuente J., Barral M., Barberan M., de Luco D.F., Blasco J.M., Gortazar C. (2010). Spatial distribution and risk factors of Brucellosis in Iberian wild ungulates. BMC Infect Dis 10: 1471-2334.

Muto M., Bazartseren B., Tsevel B., Dashzevge E., Yoshii K., Kariwa H. (2015). Isolation and characterization of tick-borne encephalitis virus from Ixodes persulcatus in Mongolia in 2012. Ticks Tick Borne Dis 19: 00088-00086.

Mutz I. (2008). Prevention. In: Compendium of Tick-borne encephalitis (TBE, FSME). Monograph, Baxter, ISW-TBE: 32-34.

Niedrig M., Avsic T., Aberle S.W., Ferenczi E., Labuda M., Rozentale B., Donoso Mantke O. (2007a). Quality control assessment for the serological diagnosis of tick borne encephalitis virus infections. J Clin Virol 38: 260-264.

Niedrig M., Donoso Mantke O., Altmann D., Zeller H. (2007b). First international diagnostic accuracy study for the serological detection of West Nile virus infection. BMC Infect Dis 7: 72.

Niedrig M., Vaisviliene D., Teichmann A., Klockmann U., Biel S.S. (2001). Comparison of six different commercial IgG-ELISA kits for the detection of TBEV-antibodies. J Clin Virol 20: 179-182.

Nuttall P.A. (1999). Pathogen-tick-host interactions: Borrelia burgdorferi and TBE virus. Zentralbl Bakteriol 289: 492-505.

Nuttall P.A., Jones L.D., Labuda M., Kaufman W.R. (1994). Adaptations of arboviruses to ticks. J Med Entomol 31: 1-9.

Obara M., Yoshii K., Kawata T., Hayasaka D., Goto A., Mizutani T., Kariwa H., Takashima I. (2006). Development of an enzyme-linked immunosorbent assay for serological diagnosis of tick-borne encephalitis using subviral particles. J Virol Methods 134: 55-60.

Obsomer V., Wirtgen M., Linden A., Claerebout E., Heyman P., Heylen D., Madder M., Maris J., Lebrun M., Tack W., Lempereur L., Hance T., Van Impe G. (2013). Spatial disaggregation of tick occurrence and ecology at a local scale as a preliminary step for spatial surveillance of tick-borne diseases: general framework and health implications in Belgium. Parasit Vectors 6: 190.

Odongo D., Kamau L., Skilton R., Mwaura S., Nitsch C., Musoke A., Taracha E., Daubenberger C., Bishop R. (2007). Vaccination of cattle with TickGARD induces cross-reactive antibodies binding to conserved linear peptides of Bm86 homologues in Boophilus decoloratus. Vaccine 25: 1287-1296.

Oehme R., Hartelt K., Backe H., Brockmann S., Kimmig P. (2002). Foci of tick-borne diseases in southwest Germany. Int J Med Microbiol 291 Suppl 33: 22-29.

OIE-WAHID. (2014). Summary on OIE-listed diseases/infections present in Belgium. Annual animal health report on the notification of the absence or presence of all diseases. http://www.oie.int/wahis_2/public/wahid.php/Reviewreport/semestrial/review?year=2014&semester=0&wild=0&country=BEL&this_country_code=BEL&detailed=1.

OIE. (2010). Training Manual on Wildlife Diseases and Surveillance. Workshop for OIE National Focal Points for Wildlife. World Organisation for Animal Health, Paris, France.: pp. 56. http://www.oie.int/fileadmin/Home/eng/Internationa_Standard_Setting/docs/pdf/WGWildlife/A_Training_Manual_Wildlife.pdf.

OIE. (2013). Rabies. In: OIE Manual of Diagnostic Tests and Vaccine for Terrestrial Animals 6th edition: pp. 307-308.

OIE. (2016). Rabies OIE Technical Disease Cards. http://www.oie.int/fileadmin/Home/eng/Media_Center/docs/pdf/Disease_cards/RABIES-EN.pdf: pp. 6.

Otranto D., de Caprariis D., Lia R.P., Tarallo V., Lorusso V., Testini G., Dantas-Torres F., Latrofa S., Diniz P.P., Mencke N., Maggi R.G., Breitschwerdt E., Capelli G., Stanneck D. (2010). Prevention of endemic canine vector-borne diseases using imidacloprid 10% and permethrin 50% in young dogs: a longitudinal field study. Vet Parasitol 172: 323-332.

Otranto D., Wall R. (2008). New strategies for the control of arthropod vectors of disease in dogs and cats. Med Vet Entomol 22: 291-302.

Palo R.T. (2014). Tick-borne encephalitis transmission risk: its dependence on host population dynamics and climate effects. Vector Borne Zoonotic Dis 14: 346-352.

Parisi S.G., Basso M., Del Vecchio C., Andreis S., Franchin E., Bello F.D., Pagni S., Biasolo M.A., Manganelli R., Barzon L., Palu G. (2016). Virological testing of cerebrospinal fluid in children aged less than 14 years with a suspected central nervous system infection: A retrospective study on 304 consecutive children from January 2012 to May 2015. Eur J Paediatr Neurol 16: 30005-30008.

Perkins S.E., Cattadori I.M., Tagliapietra V., Rizzoli A.P., Hudson P.J. (2003). Empirical evidence for key hosts in persistence of a tick-borne disease. Int J Parasitol 33: 909-917.

Pettersson J.H., Golovljova I., Vene S., Jaenson T.G. (2014). Prevalence of tick-borne encephalitis virus in Ixodes ricinus ticks in northern Europe with particular reference to Southern Sweden. Parasit Vectors 7: 102.

Pfeffer M., Dobler G. (2011). Tick-borne encephalitis virus in dogs--is this an issue? Parasit Vectors 4: 59.

Pfeffer M., Dobler G. (2013). Chapter 6: Tick-borne encephalitis. In: Beugnet, F. (Ed.) Guide to vector borne diseases of pets. Merial, Lyon, France. ISBN: 978-2-915758-40-5. : 365-379.

Piesman J. (2006). Strategies for reducing the risk of Lyme borreliosis in North America. Int J Med Microbiol 40: 17-22.

Pinter R., Madai M., Horvath G., Nemeth V., Oldal M., Kemenesi G., Dallos B., Banyai K., Jakab F. (2014). Molecular Detection and Phylogenetic Analysis of Tick-Borne Encephalitis Virus in Rodents Captured in the Transdanubian Region of Hungary. Vector Borne Zoonotic Dis 14: 621-624.

Pinter R., Madai M., Vadkerti E., Nemeth V., Oldal M., Kemenesi G., Dallos B., Gyuranecz M., Kiss G., Banyai K., Jakab F. (2013). Identification of tick-borne encephalitis virus in ticks collected in southeastern Hungary. Ticks Tick Borne Dis 4: 427-431.

Podgórski T., Baś G., Jędrzejewska B., Sönnichsen L., Śnieżko S., Jędrzejewski W., Okarma H. (2013). Spatiotemporal behavioral plasticity of wild boar (Sus scrofa) under contrasting conditions of human pressure: primeval forest and metropolitan area. Journal of Mammalogy 94: 109-119.

Pogodina V.V., Karan L.S., Koliasnikova N.M., Levina L.S., Malenko G.V., Gamova E.G., Lesnikova M.V., Kiliachina A.S., Esiunina M.S., Bochkova N.G., Shopenskaia T.A., Frolova T.V., Andaev E.I., Trukhina A.G. (2007). [Evolution of tick-borne encephalitis and a problem of evolution of its causative agent]. Vopr Virusol 52: 16-21.

Poland G.A. (2001). Prevention of Lyme disease: a review of the evidence. Mayo Clin Proc 76: 713-724.

Pound J.M., George J.E., Kammlah D.M., Lohmeyer K.H., Davey R.B. (2010). Evidence for role of white-tailed deer (Artiodactyla: Cervidae) in epizootiology of cattle ticks and southern cattle ticks (Acari: Ixodidae) in reinfestations along the Texas/Mexico border in south Texas: a review and update. J Econ Entomol 103: 211-218.

Prévot C., Licoppe A. (2013). Comparing red deer (Cervus elaphus L.) and wild boar (Sus scrofa L.) dispersal patterns in southern Belgium. European Journal of Wildlife Research 59: 795-803.

Prévot C., Morelle K. (2012). Potentiel de dispersion du sanglier et historique de la colonisation de la plaine agricoles en Wallonie. Forê 121: 35-42.

Pripuzova N.S., Gmyl L.V., Romanova L., Tereshkina N.V., Rogova Y.V., Terekhina L.L., Kozlovskaya L.I., Vorovitch M.F., Grishina K.G., Timofeev A.V., Karganova G.G. (2013). Exploring of primate models of tick-borne flaviviruses infection for evaluation of vaccines and drugs efficacy. PLoS One 8: e61094.

Progen. (2014). Immunozym FSME/TBE IgG All Species-ELISA. Data Sheet (7701075 FSME all species en_V11 - 1). Progen Biotechnik GmbH, Heidelberg, Germany. http://www.progen.de/en/immunozym-elisa-fsme-tbe-igg-all-species.html.

Progen B. (2012). Immunozym FSME IgM. Enzyme Immunoassay for the Determination of IgM-Antibodies against the TBE virus in Human

Serum, Plasma and Cerebrospinal Fluid (CSF). User's Manual Art. No; 7701045. http://www.progen.de/media/downloads/descriptions/7701045_en_V17.pdf.

Progen B.G. (2006). Immunozym FSME/TBE IgG All Species-ELISA. User's Manual. . Progen Biotechnik GmbH. Heidelberg, Germany. http://www.progen.de/apps/teProgenShop/files/products/20-218%207701075%20FSME%20all%20species%20en_13092006v8.pdf.

Puchhammer-Stockl E., Kunz C., Mandl C.W., Heinz F.X. (1995). Identification of tick-borne encephalitis virus ribonucleic acid in tick suspensions and in clinical specimens by a reverse transcription-nested polymerase chain reaction assay. Clin Diagn Virol 4: 321-326.

Pugliese A., Beltramo T., Torre D. (2007). Seroprevalence study of Tick-borne encephalitis, Borrelia burgdorferi, Dengue and Toscana virus in Turin Province. Cell Biochem Funct 25: 185-188.

Pugliese A., Gennero L., Boffito M., Vidotto V. (2002). Seroprevalence study of tick borne encephalitis in Turin province. Panminerva Med 44: 253-255.

Racz G.R., Ban E., Ferenczi E., Berencsi G. (2006). A simple spatial model to explain the distribution of human tick-borne encephalitis cases in hungary. Vector Borne Zoonotic Dis 6: 369-378.

Radda A., Kunz C., Hofmann H. (1968). [Demonstration of antibodies in sera of wild animals for the detection of foci of tick-borne encephalitis (TBE) virus in Lower Austria]. Zentralbl Bakteriol Orig 208: 88-93.

Ramelow C., Suss J., Berndt D., Roggendorf M., Schreier E. (1993). Detection of tick-borne encephalitis virus RNA in ticks (Ixodes ricinus) by the polymerase chain reaction. J Virol Methods 45: 115-119.

Randolph S. (2002). Quantitative ecology of ticks as a basis for transmission models of tick-borne pathogens. Vector Borne Zoonotic Dis 2: 209-215.

Randolph S., Green R.M. (1999). The use of satellite imagery to create dynamic risk maps for Tick-borne encephalitis. Zentralbl Bakteriol 289: 619.

Randolph S.E. (2000). Ticks and tick-borne disease systems in space and from space. Adv Parasitol 47: 217-243.

Randolph S.E. (2001). The shifting landscape of tick-borne zoonoses: tick-borne encephalitis and Lyme borreliosis in Europe. Philos Trans R Soc Lond B Biol Sci 356: 1045-1056.

Randolph S.E. (2004). Evidence that climate change has caused 'emergence' of tick-borne diseases in Europe? Int J Med Microbiol 293 Suppl 37: 5-15.

Randolph S.E. (2008). Tick-borne encephalitis incidence in Central and Eastern Europe: consequences of political transition. Microbes Infect 10: 209-216.

Randolph S.E. (2011). Transmission of tick-borne pathogens between co-feeding ticks: Milan Labuda's enduring paradigm. Ticks Tick Borne Dis 2: 179-182.

Randolph S.E., Asokliene L., Avsic-Zupanc T., Bormane A., Burri C., Gern L., Golovljova I., Hubalek Z., Knap N., Kondrusik M., Kupca A., Pejcoch M., Vasilenko V., Zygutiene M. (2008). Variable spikes in tick-borne encephalitis incidence in 2006 independent of variable tick abundance but related to weather. Parasit Vectors 1: 44.

Randolph S.E., Green R.M., Peacey M.F., Rogers D.J. (2000). Seasonal synchrony: the key to tick-borne encephalitis foci identified by satellite data. Parasitology 121: 15-23.

Randolph S.E., Miklisova D., Lysy J., Rogers D.J., Labuda M. (1999). Incidence from coincidence: patterns of tick infestations on rodents facilitate transmission of tick-borne encephalitis virus. Parasitology 118: 177-186.

Randolph S.E., Rogers D.J. (2000). Fragile transmission cycles of tick-borne encephalitis virus may be disrupted by predicted climate change. Proc Biol Sci 267: 1741-1744.

Randolph S.E., Storey K. (1999). Impact of microclimate on immature tick-rodent host interactions (Acari: Ixodidae): implications for parasite transmission. J Med Entomol 36: 741-748.

Randolph S.E., Sumilo D. (2007). Tick-borne encephalitis in Europe: dynamics of changing risk. . In: Takken W. and Knols B.G.J. (Editors), Emerging pests and vector-borne diseases in Europe First edition. Wageningen Academic Publishers, The Netherlands. : pp. 501.

Reed L.J., Muench H. (1938). A simple method of estimating fifty percent endpoints. The American Journal of Hygiene 27: 493-497.

Rees C.A. (2010). Chapter 13: Disorders of the skin - Ticks. In: Reed S.M., Bayly W.M., Sellon D.C. (Eds) Equine Internal Medicine (3rd ed), Saunders Elsevier, St. Louis, USA. ISBN: 978-1-4160-5670-6: 682-729.

Rehacek J., Kovacova E., Ciampor F., Gresikova M., Tarasevich I.V. (1987). Experimental double infection with Coxiella burnetii and tick-borne encephalitis virus in Dermacentor reticulatus ticks. Acta Virol 31: 65-73.

Reimoser F., Zandl J., Winkler D. (1999). Rehkitzmarkierung. Weidwerk 10: 26-28.

Reiner B., Fischer A. (1998). Frühsommer-Meningoenzephalitis (FSME) beim Hund in Deutschland: Zwei Fallberichte. Kleintierpraxis 43: 255-269. [in German].

Reiner B., Fischer A., Gödde T., Müller W. (1999). Clinical diagnosis of canine tickborne encephalitis (TBE): Contribution of cerebrospinal fluid analysis (CSF) and CSF antibody titers. Zbl. Bakt. 289: 605- 609.

Reiner B., Grasmück S., Steffen F., Djuric N., Schindler T., Müller W., Fischer A. (2002). Prevalence of TBE in serum and CSF of dogs with inflammatory and non-inflammatory CNS disease. Int J Med Microbiol 291(Suppl. 33): 234.

Reisen W.K. (2010). Landscape epidemiology of vector-borne diseases. Annu Rev Entomol 55: 461-483.

Remoli M.E., Marchi A., Fortuna C., Benedetti E., Minelli G., Fiorentini C., Mel R., Venturi G., Ciufolini M.G. (2015). Anti-tick-borne encephalitis (TBE) virus neutralizing antibodies dynamics in natural infections versus vaccination. Pathog Dis 73: 1-3.

Rendi-Wagner P. (2004). Risk and prevention of tick-borne encephalitis in travelers. J Travel Med 11: 307-312.

Reye A.L., Hubschen J.M., Sausy A., Muller C.P. (2010). Prevalence and seasonality of tick-borne pathogens in questing Ixodes ricinus ticks from Luxembourg. Appl Environ Microbiol 76: 2923-2931.

RicHard-59. (2011). English: Symptoms of TBE-virusinfection. . Wikipedia - Figure own work: SVG file: 751 × 552 pixels - Licensed under the Creative Commons Attribution-Share Alike 3.0 https://commons.wikimedia.org/wiki/File:TBE_symptoms.svg.

Rieger M.A., Nübling M., Huwer M., Müller W., Hofmann F. (1997). Untersuchungen zur Epidemiologie der Frühsommer-Meningoenzephalitis: Nehmen Rinder am Zyklus der Virusübertragung im südwestdeutschen Endemiegebiet teil? Erste Mitteilung. Immunität und Infektion 1: 52-57.

Rieger M.A., Nübling M., Müller W., Hasselhorn H.-M., Hofmann F., study group. (1999). Foxes as indicators for TBE endemicity – a comparative serological investigation. Extended summary. . Zentralblatt für Bakteriologie 289: 610-618.

Rieille N., Klaus C., Ambord C., Dupuis G., Péter O., Voordouw M.D.f. (2013). Use of goats as indirect indicators for detection of tick-borne encephalitis virus (TBEV) in a new risk area in Switzerland. Abstract 23rd ECMID, Berlin Germany, 27-30 April 2013 https://www.escmid.org/escmid_library/online_lecture_library

Rinaldi L., Musella V., Biggeri A., Cringoli G. (2006). New insights into the application of geographical information systems and remote sensing in veterinary parasitology. Geospat Health 1: 33-47.

Rizzoli A., Hauffe H.C., Tagliapietra V., Neteler M., Rosa R. (2009). Forest structure and roe deer abundance predict tick-borne encephalitis risk in Italy. PLoS One 4: e4336.

Rizzoli A., Neteler M., Rosa R., Versini W., Cristofolini A., Bregoli M., Buckley A., Gould E.A. (2007). Early detection of tick-borne encephalitis virus spatial distribution and activity in the province of Trento, northern Italy. Geospat Health 1: 169-176.

Rizzoli A., Rosa R., Mantelli B., Pecchioli E., Hauffe H., Tagliapietra V., Beninati T., Neteler M., Genchi C. (2004a). [Ixodes ricinus, transmitted diseases and reservoirs]. Parassitologia 46: 119-122.

Rizzoli A., Rosà R., Mantelli B., Pecchioli E., Hauffe H., Tagliapietra V., Beninati T., Neteler M., Genchi C. (2004b). Ixodes ricinus, transmitted diseases and reservoirs. Parassitologia 46(1-2): 119-122. [Article in Italian].

Rizzoli A., Silaghi C., Obiegala A., Rudolf I., Hubalek Z., Foldvari G., Plantard O., Vayssier-Taussat M., Bonnet S., Spitalska E., Kazimirova M. (2014). Ixodes ricinus and Its Transmitted Pathogens in Urban and Peri-Urban Areas in Europe: New Hazards and Relevance for Public Health. Front Public Health 2: 251.

RKI. (1998). Verbreitung der Frühsommer-Meningoenzephalitis (FSME) in Deutschland und Schlußfolgerungen für die Prävention. Robert Koch Institut Epidemiologisches Bulletin 98: p193-195. https://www.rki.de/DE/Content/Infekt/EpidBull/Archiv/1998/Ausgabenlinks/1927_1998.pdf?__blob=publicationFile.

RKI. (1999). Risikogebiete der Frühsommer-Meningoenzephalitis (FSME) in Deutschland. Robert Koch Institut Epidemiologisches Bulletin 99: p. 115. https://www.rki.de/DE/Content/Infekt/EpidBull/Archiv/1999/Ausgabenlinks/1916_1999.pdf?__blob=publicationFile.

RKI. (2001). Risikogebiete der Frühsommer-Meningoenzephalitis (FSME). Bewertung des örtlichen erkrankungsrisikos in Deutschland ermöglicht gezielte Prävention für Exponierte. Robert Koch Institut Epidemiologisches Bulletin 2001: p. 105-109. http://www.rki.de/DE/Content/Infekt/EpidBull/Archiv/2001/Anlagen/2016_KARTE_2001_pdf.html?nn=2375018.

RKI. (2002). Risikogebiete der Frühsommer-Meningoenzephalitis (FSME) in Deutschland. Bewertung des örtlichen Erkrankungsrisikos ermöglicht gezielte Prävention für Exponiert. Robert Koch Institut Epidemiologisches Bulletin 2002: p. 212-215. http://www.rki.de/DE/Content/Infekt/EpidBull/Archiv/2002/Ausgabenlinks/2026_2002.pdf?__blob=publicationFile.

RKI. (2003). Risikogebiete der Frühsommer-Meningoenzephalitis (FSME) in Deutschland: Aktualisierte Darstellung auf der Basis der Daten des Jahres 2002 (Stand: 31.5.2003). . Robert Koch Institut Epidemiologisches Bulletin 20: 1-3. http://www.rki.de/cln_151/nn_196440/DE/Content/Infekt/EpidBull/Archiv/192003/196420__196403,templateId=raw,property=publicationFile.pdf/196420_196403.pdf.

RKI. (2004). Risikogebiete der Frühsommer-Meningoenzephalitis (FSME) in Deutschland. Bewertung des örtlichen Erkrankungsrisikos ermöglicht gezielte Prävention. Robert Koch Institut Epidemiologisches Bulletin 2004: p. 169-173. http://www.rki.de/DE/Content/Infekt/EpidBull/Archiv/2004/Ausgabenlinks/2021_2004.pdf?__blob=publicationFile.

RKI. (2005). FSME: Risikogebiete in Deutschland. Zum örtlichen Erkrankungsrisiko der Frühsommer-Meningoenzephalitis und zu Schlussfolgerungen für präventive Maßnahme. Robert Koch Institut Epidemiologisches Bulletin 2005: p. 137-148. https://www.rki.de/DE/Content/Infekt/EpidBull/Archiv/2005/Ausgabenlinks/2016_2005.html.

RKI. (2006). FSME: Zum aktuellen Vorkommen in Deutschland Bewertung des örtlichen Erkrankungsrisikos – Karte der Risikogebiete. Robert Koch Institut Epidemiologisches Bulletin 2006: p.129 -133. http://www.rki.de/DE/Content/Infekt/EpidBull/Archiv/2006/Ausgabenlinks/2017_2006.pdf?__blob=publicationFile.

RKI. (2007). FSME: Risikogebiete in Deutschland.Bewertung des örtlichen erkrankungsrisikos – Karte der Risikogebiete. Robert Koch Institut Epidemiologisches Bulletin 2007: p.129-140. https://www.rki.de/DE/Content/Infekt/EpidBull/Archiv/2007/Ausgaben/Tabelle_2015_2007.html.

RKI. (2009). FSME: Risikogebiete in Deutschland. Bewertung des örtlichen Erkrankungsrisikos. . Robert Koch Institut Epidemiologisches Bulletin 18: p. 165-172. http://www.rki.de/DE/Content/Infekt/EpidBull/Archiv/2009/Ausgaben/2018_2009.pdf?__blob=publicationFile.

RKI. (2010). FSME: Risikogebiete in Deutschland (Stand: April 2010). Bewertung des örtlichen Erkrankungsrisiko. Robert Koch Institut Epidemiologisches Bulletin 2010: p. 147-155. http://www.rki.de/DE/Content/Infekt/EpidBull/Archiv/2010/Ausgaben/2017_2010.pdf?__blob=publicationFile.

RKI. (2011). FSME: Risikogebiete in Deutschland (Stand: April 2011). Bewertung des örtlichen Erkrankungsrisikos Bulletin Robert Koch Institut Epidemiologisches Bulletin 2011: p133-148. https://www.rki.de/DE/Content/Infekt/EpidBull/Archiv/2011/Ausgaben/2017_2011.pdf?__blob=publicationFile.

RKI. (2012). FSME: Risikogebiete in Deutschland (Stand: Mai 2012). Bewertung des örtlichen Erkrankungsrisiko Robert Koch Institut Epidemiologisches Bulletin 2012: p. 189-200. http://www.rki.de/DE/Content/Infekt/EpidBull/Archiv/2012/Ausgaben/2021_2012.pdf?__blob=publicationFile.

RKI. (2013). FSME: Risikogebiete in Deutschland (Stand: Mai 2013). Bewertung des örtlichen Erkrankungsrisikos Robert Koch Institut Epidemiologisches Bulletin 2013: p. 151-162. http://www.rki.de/DE/Content/Infekt/EpidBull/Archiv/2013/Ausgaben/2018_2013.pdf?__blob=publicationFile.

RKI. (2014). FSME Risikogebiete in Deutschland (Stand: April 2014). Bewertung des örtlichen erkrankungs-risikos Epidemiologisches Bulletin 2014: p. 121– 133. http://www.rki.de/DE/Content/Infekt/EpidBull/Archiv/2014/Ausgaben/2015_2014.pdf?__blob=publicationFile.

RKI. (2015). FSME: Risikogebiete in Deutschland (Stand: Mai 2015). Bewertung des örtlichen Erkrankungsrisikos. Robert Koch Institut Epidemiologisches Bulletin 2015: p. 175-186. http://www.rki.de/DE/Content/InfAZ/F/FSME/Karte_FSME.pdf?__blob=publicationFile.

Robin X., Turck N., Hainard A., Tiberti N., Lisacek F., Sanchez J.-C., Müller M. (2011). pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinformatics 12: 77.

Rodriguez-Prieto V., Vicente-Rubiano M., Sanchez-Matamoros A., Rubio-Guerri C., Melero M., Martinez-Lopez B., Martinez-Aviles M., Hoinville L., Vergne T., Comin A., Schauer B., Dorea F., Pfeiffer D.U., Sanchez-Vizcaino J.M. (2014). Systematic review of surveillance systems and methods for early detection of exotic, new and re-emerging diseases in animal populations. Epidemiol Infect: 1-25.

Roelandt S., Heyman P., De Filette M., Vene S., Van der Stede Y., Caij A.B., Tavernier P., Dobly A., De Bosschere H., Vyt P., Meersschaert C., Roels S. (2011). Tick-borne encephalitis virus seropositive dog detected in Belgium: screening of the canine population as sentinels for public health. Vector Borne Zoonotic Dis 11: 1371-1376.

Roelandt S., Heyman P., Tavernier P., Roels S. (2010). Tick-borne encephalitis in Europe: Review of an emerging zoonosis. Tekenencefalitis in Europa: overzicht van een opkomende zoönose. . Vlaams Diergeneeskundig Tijdschrift 79: pp 23-31

Roelandt S., Suin V., Riocreux F., Lamoral S., Van der Heyden S., Van der Stede Y., Lambrecht B., Caij B., Brochier B., Roels S., Van Gucht S. (2014). Autochthonous tick-borne encephalitis virus-seropositive cattle in belgium: a risk-based targeted serological survey. Vector Borne Zoonotic Dis 14: 640-647.

Roelandt S., Suin V., Van der Stede Y., Lamoral S., Marche S., Tignon M., Saiz J.-C., Escribano-Romero E., Casaer J., Brochier B., Van Gucht S., Roels S., Vervaeke M. (2016). First TBEV serology screening of Flemish wild boar. Infection Ecology and Epidemiology 6: 31099. http://dx.doi.org/31010.33402/iee.v31096.31099.

Rogers D.J., Hay S.I., Packer M.J. (1996). Predicting the distribution of tsetse flies in West Africa using temporal Fourier processed meteorological satellite data. Annals of Tropical Medicine and Parasitology 90: 225-241.

Roggendorf M., Girgsdies O.E., Rosenkranz G. (1994). Epidemiologie und Prophylaxe der Friihsommer-Meningoenzephalitis. . Die Gelben Hefte 34: 74-80.

Romanova L., Gmyl A.P., Dzhivanian T.I., Bakhmutov D.V., Lukashev A.N., Gmyl L.V., Rumyantsev A.A., Burenkova L.A., Lashkevich V.A., Karganova G.G. (2007). Microevolution of tick-borne encephalitis virus in course of host alternation. Virology 362: 75-84.

Rosseels V., Naze F., De Craeye S., Francart A., Kalai M., Van Gucht S. (2011). A non-invasive intranasal inoculation technique using isoflurane anesthesia to infect the brain of mice with rabies virus. J Virol Methods 173: 127-136.

Rushton J.O., Lecollinet S., Hubalek Z., Svobodova P., Lussy H., Nowotny N. (2013). Tick-borne encephalitis virus in horses, Austria, 2011. Emerg Infect Dis 19: 635-637.

Ruzek D., Bilski B., Günther G. (2013). Chapter 10: Tick-Borne Encephalitis. In: S.K. Singh, D. Ruzek (editors) Neuroviral Infections: RNA Viruses and Retroviruses - CRC Press, USA: p. 211-238.

Ruzek D., Piskunova N., Zampachova E. (2007a). High variability in viral load in cerebrospinal fluid from patients with herpes simplex and varicella-zoster infections of the central nervous system. Clin Microbiol Infect 13: 1217-1219.

Ruzek D., Stastna H., Kopecky J., Golovljova I., Grubhoffer L. (2007b). Rapid subtyping of tick-borne encephalitis virus isolates using multiplex RT-PCR. J Virol Methods 144: 133-137.

RW. (2009). Natura2000 espèces & habitats protégés. Région Wallonne.

Ryser-Degiorgis M.P. (2013). Wildlife health investigations: needs, challenges and recommendations. BMC Vet Res 9: 1746-6148.

Saegerman C., Claerebout E., Kalume M., Losson B. (2007). Bovine babesiosis in Belgium: preliminary results of postal survey of veterinarians in 2006. Renc. Rech. Ruminants. 14: 220.

Saksida A., Duh D., Lotric-Furlan S., Strle F., Petrovec M., Avsic-Zupanc T. (2005). The importance of tick-borne encephalitis virus RNA detection for early differential diagnosis of tick-borne encephalitis. J Clin Virol 33: 331-335.

Sandvik B. (2009). World Borders Dataset. TM_WORLD_BORDERS-0.3.zip. http://thematicmapping.org/downloads/world_borders.php.

Sargeant J.M., Kelton D.F., O'Connor A.M. (2014). Study designs and systematic reviews of interventions: building evidence across study designs. Zoonoses Public Health 1: 10-17.

SAS. (2008). Existence of Maximum Likelihood Estimates. The LOGISTIC Procedure (Book Excerpt). In: SAS/STAT®9.2 User's Guide. Second Edition, SAS Institute Inc., USA. p.75.

https://support.sas.com/documentation/cdl/en/statuglogistic/61802/PDF/default/statuglogistic.pdf.

Scheppers T., Casaer J., Vercammen J., Wils C. (2011). Afbakening van beheerzones voor everzwijn in Vlaanderen. (INBO.R.2011.24 - D/2011/3241/208). (Report in Dutch). Instituut voor Natuur- en Bosonderzoek, Brussel pp. 83.

Scheppers T., Huysentruyt F., Neukermans A., Vercammen J., Verschaffel E., Casaer J. (2013). Grofwildjacht in Vlaanderen - Cijfers en statistieken over de periode 2002 - 2012. (INBO.R.2013.30 - D/2013/3241/185). (Report in Dutch – English abstract). Instituut voor Natuur- en Bosonderzoek: pp. 93.

Scheppers T., Huysentruyt F., Neukermans A., Vercammen J., Verschaffel E., Casaer J. (2014). Grofwildjacht in Vlaanderen - Cijfers en statistieken 2013. Mededelingen van

het Instituut voor Natuur- en Bosonderzoek 2014 (INBO.M.2014.2520956). (Report in Dutch) Instituut voor Natuur- en Bosonderzoek, Brussel.

Schneider H. (1931). Über epidemische Meningitis serosa. Wien Klin Wschr. 44: 350.

Schreiber C., Krucken J., Beck S., Maaz D., Pachnicke S., Krieger K., Gross M., Kohn B., von Samson-Himmelstjerna G. (2014). Pathogens in ticks collected from dogs in Berlin/Brandenburg, Germany. Parasit Vectors 7: 014-0535.

Schuijt T.J., Hovius J.W., van der Poll T., van Dam A.P., Fikrig E. (2011). Lyme borreliosis vaccination: the facts, the challenge, the future. Trends Parasitol 27: 40-47.

Schwaiger M., Cassinotti P. (2003). Development of a quantitative real-time RT-PCR assay with internal control for the laboratory detection of tick borne encephalitis virus (TBEV) RNA. J Clin Virol 27: 136-145.

Shaw S. (2005). Chapter 13: Other Arthropod-borne infectious diseases of the dog and cat. In: Shaw S., Day, M. (Eds) Arthropod-borne Infectious Diseases of the Dog and Cat. CRC Press, Taylor and Francis Group, USA. ISBN: 978-1-84076-578-6. . p.138-142.

Shope R.E., Sather G.E. (1979). Arboviruses. In: Lennette EH, Schmidt NJ, eds. Diagnostic procedures for viral, rickettsial and chlamydial infections 5th ed. Washington DC, USA: Am Publ Hlth Ass: 767-814.

Sibbald S., Carter P., Poulton S. (2006). Proposal for a National Monitoring Scheme for Small Mammals in the United Kingdom and the Republic of Eire. The Mammal Society Research Report No. 6.

Siemens. (2016). BEP III Infectious Disease Testing System. Enzygnost Assays. http://www.healthcare.siemens.com/infectious-disease-testing/systems/bep-iii-system/assays.

Sîkutova S., Hornok S., Hubalek Z., Dolezalkova I., Juricova Z., Rudolf I. (2009). Serological survey of domestic animals for tick-borne encephalitis and Bhanja viruses in northeastern Hungary. Vet Microbiol 135: 267-271.

Šimundić A.-M. (2008). Measures of diagnostic accuracy: basic definitions. eJIFCC - electronic Journal of the IFCC: International Federation of Clinical Chemistry and Laboratory Medicine 19 pp. 9. http://www.ifcc.org/ifcc-communications-publications-division-%28cpd%29/ifcc-publications/ejifcc-%28journal%29/e-journal-volumes/ejifcc-2008-vol-2019/vol-2019-no-2004/measures-of-diagnostic-accuracy-basic-definitions/.

Sixl W., Batikova M., Stunzner D., Sekeyova M., Sixl-Voigt B., Gresikova M. (1973). Haemagglutination-inhibiting antibodies against arboviruses in animal sera, collected in some regions in Austria. II. Zentralbl Bakteriol Orig A 224: 303-308.

Skarpaas T., Golovljova I., Vene S., Ljostad U., Sjursen H., Plyusnin A., Lundkvist A. (2006). Tickborne encephalitis virus, Norway and Denmark. Emerg Infect Dis 12: 1136-1138.

Skarpaas T., Ljostad U., Sundoy A. (2004). First human cases of tickborne encephalitis, Norway. Emerg Infect Dis 10: 2241-2243.

Skarpaas T., Sundoy A., Bruu A.L., Vene S., Pedersen J., Eng P.G., Csango P.A. (2002). [Tick-borne encephalitis in Norway]. Tidsskr Nor Laegeforen 122: 30-32.

Skarphedinsson S., Jensen P.M., Kristiansen K. (2005). Survey of tickborne infections in Denmark. Emerg Infect Dis 11: 1055-1061.

Smith M.O. (2002). Chapter 33. Diseases of the Nervous System. . In: Smith B.P. (ed) Large Animal Internal Medicine, 3rd Edition Mosby Harcourt, St. Louis, Missouri, USA. ISBN: 9780323009461.

Solomon T., Hart I.J., Beeching N.J. (2007). Viral encephalitis: a clinician's guide. Practical Neurology 7: 288-305. doi:210.1136/jnnp.2007.129098.

Sonenshine D.E., Mather T.N. (1994). Micrometeorological and Microhabitat Factors. Ecological Dynamics of Tick-Borne Zoonoses. Oxford University Press, 20 Oct 1994 - Science. ISBN 0-19-507313-4: pp. 464.

Sonnenberg K., Niedrig M., Steinhagen K., Rohwader E., Meyer W., Schlumberger W., Muller-Kunert E., Stocker W. (2004). State-of-the-art serological techniques for detection of antibodies against tick-borne encephalitis virus. Int J Med Microbiol 293 Suppl 37: 148-151.

Southwood T.R.E., Henderson P. (2000). Ecological Methods. Blackwell Science 3rd edn, Oxford. ISBN: 978-0-632-05477-0. pp. 592.

Stadtbaumer K., Leschnik M.W., Nell B. (2004). Tick-borne encephalitis virus as a possible cause of optic neuritis in a dog. Vet Ophthalmol 7: 271-277.

Stafford K.C., 3rd. (1991). Effectiveness of host-targeted permethrin in the control of Ixodes dammini (Acari: Ixodidae). J Med Entomol 28: 611-617.

Stafford K.C., 3rd, Denicola A.J., Pound J.M., Miller J.A., George J.E. (2009). Topical treatment of white-tailed deer with an acaricide for the control of Ixodes scapularis (Acari: Ixodidae) in a Connecticut Lyme borreliosis hyperendemic Community. Vector Borne Zoonotic Dis 9: 371-379.

Stafford K.C.r. (2004). Tick Management Handbook: A integrated guide for homeowners, pest control operators, and public health officials for the prevention of tick-associated disease. The Connecticut Agricultural Experiment Station, New Haven, Connecticut, USA http://www.ct.gov/caes/lib/caes/documents/special_features/tickhandbook.pdf: pp.71.

Stage B. (1992). Untersuchung an Rehseren aus dem Landkreis Tiibingen auf Antikorper gegen Borrelia burgdorferi [Inaugural-Dissertation]. Tiubingen.

Stefanoff P., Pfeffer M., Hellenbrand W., Rogalska J., Ruhe F., Makowka A., Michalik J., Wodecka B., Rymaszewska A., Kiewra D., Baumann-Popczyk A., Dobler G. (2013). Virus detection in questing ticks is not a sensitive indicator for risk assessment of tick-borne encephalitis in humans. Zoonoses Public Health 60: 215-226.

Stefanoff P., Polkowska A., Giambi C., Levy-Bruhl D., O'Flanagan D., Dematte L., Lopalco P.L., Mereckiene J., Johansen K., D'Ancona F. (2011). Reliable surveillance of tick-borne encephalitis in European countries is necessary to improve the quality of vaccine recommendations. Vaccine 29: 1283-1288.

Steffen R. (2009). Ups and downs with TBE in Switzerland. In: Proceedings of the International Conference: Climate change impact on ticks and tick-borne diseases Brussels, Belgium 2/2/2009.

Steffen R. (2016). Epidemiology of tick-borne encephalitis (TBE) in international travellers to Western/Central Europe and conclusions on vaccination recommendations. J Travel Med 23.

Stevenson M., Nunes T., Heuer C., Marshall J., Sanchez J., Thornton R., Reiczigel J., Robison-Cox J., Sebastiani P., Solymos P., Yoshida K. (2014). epiR: An R package for the analysis of epidemiological data. R package version 0.9-58. http://CRAN.R-project.org/package=epiR

Stjernberg L., Holmkvist K., Berglund J. (2008). A newly detected tick-borne encephalitis (TBE) focus in south-east Sweden: a follow-up study of TBE virus (TBEV) seroprevalence. Scand J Infect Dis 40: 4-10.

Storch G.A. (2007). Chapter 17: Diagnostic Virology. In: (Eds. Knipe D.M., Howley P.M.) Fields Virology Fifth Edition Volume One: 592-593.

Süss J. (2003). Epidemiology and ecology of TBE relevant to the production of effective vaccines. Vaccine 21, Supplement 1: S19-S35.

Süss J. (2008a). Epidemiology. . Compendium of Tick-borne encephalitis (TBE, FSME). Monograph,: 15-20.

Süss J. (2008b). Tick-borne encephalitis in Europe and beyond--the epidemiological situation as of 2007. Euro Surveill 13.

Süss J. (2011). Tick-borne encephalitis 2010: epidemiology, risk areas, and virus strains in Europe and Asia-an overview. Ticks Tick Borne Dis 2: 2-15.

Süss J., Béziat P., Schrader C. (1997). Viral zoonosis from the viewpoint of their epidemiological surveillance: tick-borne encephalitis as a model. Arch Virol Suppl 13: 229-243.

Süss J., Gelpi E., Klaus C., Bagon A., Liebler-Tenorio E.M., Budka H., Stark B., Müller W., Hotzel H. (2007). Tickborne encephalitis in naturally exposed monkey (*Macaca sylvanus*). Emerg Infect Dis 13: 905-907.

Süss J., Kahl O., Aspock H., Hartelt K., Vaheri A., Oehme R., Hasle G., Dautel H., Kunz C., Kupreviciene N., Zimmermann H.P., Atkinson B., Dobler G., Kutsar K., Heinz F.X. (2010). Tick-borne encephalitis in the age of general mobility. Wien Med Wochenschr 160: 94-100.

Süss J., Klaus C., Diller R., Schrader C., Wohanka N., Abel U. (2006). TBE incidence versus virus prevalence and increased prevalence of the TBE virus in Ixodes ricinus removed from humans. Int J Med Microbiol 40: 63-68.

Süss J., Schrader C., Abel U., Bormane A., Duks A., Kalnina V. (2002). Characterization of tick-borne encephalitis (TBE) foci in Germany and Latvia (1997-2000). Int J Med Microbiol 291 Suppl 33: 34-42.

Süss J., Schrader C., Abel U., Voigt W.P., Schosser R. (1999). Annual and seasonal variation of tick-borne encephalitis virus (TBEV) prevalence in ticks in selected hot spot areas in Germany using a nRT-PCR: results from 1997 and 1998. Zentralbl Bakteriol 289: 564-578.

Süss J., Schrader C., Falk U., Wohanka N. (2004). Tick-borne encephalitis (TBE) in Germany--epidemiological data, development of risk areas and virus prevalence in field-collected ticks and in ticks removed from humans. Int J Med Microbiol 293 Suppl 37: 69-79.

Süss J., Sinnecker H., Sinnecker R., Berndt D., Zilske E., Dedek G., Apitzsch L. (1992). Epidemiology and ecology of tick-borne encephalitis in the eastern part of Germany between 1960 and 1990 and studies on the dynamics of a natural focus of tick-borne encephalitis. Zentralbl Bakteriol 277: 224-235.

Svoboda P., Dobler G., Markotic A., Kurolt I.C., Speck S., Habus J., Vucelja M., Krajinovic L.C., Tadin A., Margaletic J., Essbauer S. (2014). Survey for Hantaviruses, Tick-Borne Encephalitis Virus, and Rickettsia spp. in Small Rodents in Croatia. Vector Borne Zoonotic Dis 14: 523-530.

Tack W., Madder M., Baeten L., De Frenne P., Verheyen K. (2012). The abundance of Ixodes ricinus ticks depends on tree species composition and shrub cover. Parasitology 139: 1273-1281.

Takashima I. (1998). Epidemiology of tick-borne encephalitis in Japan. Comp Immunol Microbiol Infect Dis 21: 81-90.

Takashima I., Hayasaka D., Goto A., Kariwa H., Mizutani T. (2001). Epidemiology of tick-borne encephalitis (TBE) and phylogenetic analysis of TBE viruses in Japan and Far Eastern Russia. Jpn J Infect Dis 54: 1-11.

Takashima I., Morita K., Chiba M., Hayasaka D., Sato T., Takezawa C., Igarashi A., Kariwa H., Yoshimatsu K., Arikawa J., Hashimoto N. (1997). A case of tick-borne encephalitis in Japan and isolation of the the virus. J Clin Microbiol 35: 1943-1947.

Takashima I., Ueda M., Kiyotake M., Furuta I., Hashimoto N. (1992). Epidemiology of tick borne encephalitis. J. Vet. Med. 45: 831–835. [In Japanese].

Takeda T., Ito T., Chiba M., Takahashi K., Niioka T., Takashima I. (1998). Isolation of tick-borne encephalitis virus from Ixodes ovatus (Acari: Ixodidae) in Japan. J Med Entomol 35: 227-231.

Takeda T., Ito T., Osada M., Takahashi K., Takashima I. (1999). Isolation of tick-borne encephalitis virus from wild rodents and a seroepizootiologic survey in Hokkaido, Japan. Am J Trop Med Hyg 60: 287-291.

Takezawa C., Sato T., Mizutani Y., Abe S., Morita K. (1995). Russian spring-summer encephalitis. A case report. Neurological Medicine 43: 251-255.

Tavernier P., Sys S.U., De Clerck K., De Leeuw I., Caij A.B., De Baere M., De Regge N., Fretin D., Roupie V., Govaerts M., Heyman P., Vanrompay D., Yin L., Kalmar I., Suin V., Brochier B., Dobly A., De Craeye S., Roelandt S., Goossens E., Roels S. (2015). Serologic screening for thirteen infectious agents in roe deer (Capreolus capreolus) in Flanders. Infection Ecology and Epidemiology 5: pp. 12. http://www.infectionecologyandepidemiology.net/index.php/iee/article/view/29862/pdf_29832.

Taylor M.A. (2012). Ectoparasiticides Used in Large Animals. In: The Merck Veterinary Manual Online http://wwhttp://www.merckmanuals.com/vet/pharmacology/ectoparasiticides/ectoparasiticides_used_in_large_animals.html?qt=tick%20repellent%20large%20animals&alt=sh.

Ternovoi V.A., Protopopova E.V., Chausov E.V., Novikov D.V., Leonova G.N., Netesov S.V., Loktev V.B. (2007). Novel variant of tickborne encephalitis virus, Russia. Emerg Infect Dis 13: 1574-1578.

Testline. (2015). Enzyme Immunoassays for the diagnosis of tick-borne encephalitis. www.testlinecd.com/file/1555/TBEV.pdf.

Thein P. (2009). Durch Biovektoren übertragene Infektionen des Pferdes:

Faktoren, Vektoren, Erreger. Pferdeheilkunde 25(4) 345-353.

Thiry D., Mauroy A., Saegerman C., Licoppe A., Fett T., Thomas I., Brochier B., Thiry E., Linden A. (2015). Belgian Wildlife as Potential Zoonotic Reservoir of Hepatitis E Virus. Transbound Emerg Dis 31: 12435.

Thomas L., Buckland S.T., Rexstad E.A., Laake J.L., Strindberg S., Hedley S.L., Bishop J.R.B., Marques T.A., Burnham K.P. (2010). Distance software: design and analysis of distance sampling surveys for estimating population size. Journal of Applied Ecology 47: 5-14.

Thrusfield M., Ortega C., de Blas I., Noordhuizen J.P., Frankena K. (2001). WIN EPISCOPE 2.0: improved epidemiological software for veterinary medicine. Vet Rec 148: 567-572.

Thurmond M.C. (2003). Conceptual foundations for infectious disease surveillance. J Vet Diagn Invest 15: 501-514.

Tipold A., Fatzer R., Holzmann H. (1993). Zentraleuropäische Zeckenenzephalitis beim Hund. Kleintierpraxis 38: 619-628.

Tonteri E., Jaaskelainen A.E., Tikkakoski T., Voutilainen L., Niemimaa J., Henttonen H., Vaheri A., Vapalahti O. (2011). Tick-borne encephalitis virus in wild rodents in winter, Finland, 2008-2009. Emerg Infect Dis 17: 72-75.

Tonteri E., Kipar A., Voutilainen L., Vene S., Vaheri A., Vapalahti O., Lundkvist A. (2013). The three subtypes of tick-borne encephalitis virus induce encephalitis in a natural host, the bank vole (Myodes glareolus). PLoS One 8: e81214.

Treib J., Haass A., Woessner R., Grauer M.T., Schimrigk K., Kießig S.T. (1996). Tick-borne encephalitis diagnosis in patients with inflammatory changes in the cerebrospinal fluid in a region with very low prevalence. Infection 24: 400-402.

Trimnell A.R., Davies G.M., Lissina O., Hails R.S., Nuttall P.A. (2005). A cross-reactive tick cement antigen is a candidate broad-spectrum tick vaccine. Vaccine 23: 4329-4341.

Truvé J., Lemel J., Söderberg B. (2004). Dispersal in relation to population density in wild boar (Sus scrofa). Galemys 16: 75-82.

Tunkel A.R., Glaser C.A., Bloch K.C., Sejvar J.J., Marra C.M., Roos K.L., Hartman B.J., Kaplan S.L., Scheld W.M., Whitley R.J. (2008). The management of encephalitis: clinical practice guidelines by the Infectious Diseases Society of America. Clin Infect Dis 47: 303-327.

Uspensky I. (1996). Tick-borne encephalitis prevention through vector control in Russia: an historical review. Review of Medical and Veterinary Entomology 84(10): 679-689.

Vaillant V., teams T.F.a.B.m.i. (2008). Identification of a rabid dog illegally introduced from the Republic of the Gambia to Belgium and France. Euro Surveill 13.

Van Den Berge K., Pauw W. (2003). Vos - Vulpes vulpes (Linnaeus, 1758). In: Verkem, S., De Maeseneer, J., Vandendriessche, B., Verbeylen, G. & Yskout, S. Zoogdieren in Vlaanderen. Ecologie en verspreiding van 1987 tot 2002. Natuurpunt Studie en JNM-Zoogdierenwerkgroep, Mechelen en Gent, België. http://waarnemingen.be/soort/info/428.

van der Poel W.H., Van der Heide R., Bakker D., De Looff M., De Jong J., Van Manen N., Gaasenbeck C.P., Borgsteede F.H. (2005). Attempt to detect evidence for tick-borne encephalitis virus in ticks and mammalian wildlife in The Netherlands. Vector Borne Zoonotic Dis 5: 58-64.

Van der Stede Y., Cox E., Verdonck F., Vancaeneghem S., Goddeeris B.M. (2003). Reduced faecal excretion of F4+-E coli by the intramuscular immunisation of suckling piglets by the addition of 1alpha,25-dihydroxyvitamin D3 or CpG-oligodeoxynucleotides. Vaccine 21: 1023-1032.

Van Gucht S., Le Roux I. (2008). Rabies control in Belgium: from eradication in foxes to import of a contaminated dog. Vlaams Diergeneeskundig Tijdschrift 77: 376-384. http://vdt.ugent.be/sites/default/files/art77602.pdf.

Van Herzele A., Aarts N., Casaer J. (2015). To protect or to kill? Polarising dynamics of public debate on foxes and wild boar in Flanders. [Poster]. https://data.inbo.be/purews/files/8735566/To_protect_or_to_kill_Polarising_dynamics_of_public_debate_on_foxes_and_wild_boar_in_Flanders_FP.pdf.

Vanwambeke S.O., Sumilo D., Bormane A., Lambin E.F., Randolph S.E. (2010). Landscape predictors of tick-borne encephalitis in Latvia: land cover, land use, and land ownership. Vector Borne Zoonotic Dis 10: 497-506.

VBORNET. (2015). Ixodes ricinus - current known distribution - October 2015. Tick species - Distribution maps ECDC-EFSA 2015/VECTORNET. http://ecdc.europa.eu/en/healthtopics/vectors/vector-maps/Pages/VBORNET-maps-tick-species.aspx.

Vene S., Haglund M., Vapalahti O., Lundkvist A. (1998). A rapid fluorescent focus inhibition test for detection of neutralizing antibodies to tick-borne encephalitis virus. J Virol Methods 73: 71-75.

Venturi G., Martelli P., Mazzolini E., Fiorentini C., Benedetti E., Todone D., Villalta D., Fortuna C., Marchi A., Minelli G., Ciufolini M.G. (2009). Humoral immunity in natural infection by tick-borne encephalitis virus. J Med Virol 81: 665-671.

Venturi G., Mel R., Marchi A., Mancuso S., Russino F., Pra G.D., Papa N., Bertiato G., Fiorentini C., Ciufolini M.G. (2006). Humoral immunity and correlation between ELISA, hemagglutination inhibition, and neutralization tests after vaccination against tick-borne encephalitis virus in children. J Virol Methods 134: 136-139.

Verkem S., De Maeseneer J., Vandendriessche B., Verbeylen G., Yskout S. (2003). Zoogdieren in Vlaanderen. Ecologie en verspreiding van 1987 tot 2002. Natuurpunt Studie & JNM-Zoogdierenwerkgroep Mechelen & Gent, België.

Verraes C., Vlaemynck G., Van Weyenberg S., De Zutter L., Daube G., Sindic M., Uyttendaele M., Herman L. (2015). A review of the microbiological hazards of dairy products made from raw milk. International Dairy Journal 50: 32-44.

Vervaeke M. (2012). Zijn de everzwijnen in Vlaanderen gezond ? Overzicht van de surveillance van Klassieke varkenspest, Brucellose en ziekte van Aujeszky bij in het wild levende everzwijnen in het Vlaamse Gewest in de periode 2010-2012. (Report In Dutch). pp. 7. http://www.natuurenbos.be/nl-BE/natuurbeleid/soortenbeleid/ziekten.

Vesenjak-Hirjan J., Galinovic-Weisglass M., Brudnjak Z. (1976a). Infections with tick-borne encephalitis virus in the Mediterranean focus Nadsela (Island of Brac). Rad jugoslavenska akademije znanosti umjetnosti 13: 29-36.

Vesenjak-Hirjan J., Galinovic-Weisglass M., Brudnjak Z. (1976b). Infections with tick-borne encephalitis virus in the Pannonian focus Stara Ves. 1. Serological studies 1961-1964. Rad jugoslavenska akademije znanosti umjetnosti 13: 11-20.

Vesenjak-Hirjan J., Galinovic-Weisglass M., Brudnjak Z. (1976c). Infections with tick-borne encephalitis virus in the Pannonian focus Stara Ves. 2. Serological studies 1972. Rad jugoslavenska akademije znanosti umjetnosti 13 21-28.

Vesenjak-Hirjan J., Galinovic-Weisglass M., Brudnjak Z. (1976d). Infections with tick-borne encephalitis virus in the Pannonian focus Stara Ves. 3. Persistence of HI, CF and N antibodies to TBE virus. Rad jugoslavenska akademije znanosti umjetnosti 13 29-36.

Vicente J., Hofle U., Garrido J.M., Fernandez-de-Mera I.G., Acevedo P., Juste R., Barral M., Gortazar C. (2007). Risk factors associated with the prevalence of tuberculosis-like lesions in fenced wild boar and red deer in south central Spain. Vet Res 38: 451-464.

Vicente J., Hofle U., Garrido J.M., Fernandez-De-Mera I.G., Juste R., Barral M., Gortazar C. (2006). Wild boar and red deer display high prevalences of tuberculosis-like lesions in Spain. Vet Res 37: 107-119.

Waldenström J., Lundkvist A., Falk K.I., Garpmo U., Bergstrom S., Lindegren G., Sjostedt A., Mejlon H., Fransson T., Haemig P.D., Olsen B. (2007). Migrating birds and tickborne encephalitis virus. Emerg Infect Dis 13: 1215-1218.

Walder G., Lkhamsuren E., Shagdar A., Bataa J., Batmunkh T., Orth D., Heinz F.X., Danichova G.A., Khasnatinov M.A., Wurzner R., Dierich M.P. (2006). Serological evidence for tick-borne encephalitis, borreliosis, and human granulocytic anaplasmosis in Mongolia. Int J Med Microbiol 40: 69-75.

Waldvogel A., Matile H., Wegmann C., Wyler R., Kunz C. (1981). [Tick-borne encephalitis in the horse]. Schweiz Arch Tierheilkd 123: 227-233.

Walker A.R. (2011). Eradication and control of livestock ticks: biological, economic and social perspectives. Parasitology 138: 945-959.

Wallner G., Mandl C.W., Ecker M., Holzmann H., Stiasny K., Kunz C., Heinz F.X. (1996). Characterization and complete genome sequences of high- and low- virulence variants of tick-borne encephalitis virus. J Gen Virol 77 (Pt 5): 1035-1042.

Wandeler A., Steck F., Fankhauser R., Kammermann B., Gresikova M., Blascovic D. (1972). [Isolation of the virus of Central European tick-borne encephalitis in Switzerland]. Pathol Microbiol (Basel) 38: 258-270.

Warns-Petit E., Artois M., Calavas D. (2009). Biosurveillance de la faune sauvage (Wildlife biosurveillance). Bulletin de l'Académie vétérinaire de France 162: 205-213. http://www.academie-veterinaire-defrance.org.

Wattle O. (1992). Tick-borne encephalitis virusinfektion hos hund. Fördjupninsarbete, Vet. Med. Fakulteten SLU. [in Swedish].

Weissbach F.H., Hirsch H.H. (2015). Comparison of Two Commercial Tick-Borne Encephalitis Virus IgG Enzyme-Linked Immunosorbent Assays. Clin Vaccine Immunol 22: 754-760.

Weissenbock H., Hubalek Z., Bakonyi T., Nowotny N. (2010). Zoonotic mosquito-borne flaviviruses: worldwide presence of agents with proven pathogenicity and potential candidates of future emerging diseases. Vet Microbiol 140: 271-280.

Weitlauf J.C., Ruzek J.I., Westrup D.A., Lee T., Keller J. (2007). Empirically Assessing Participant Perceptions of the Research Experience in a Randomized Clinical Trial: The Women's Self-Defense Project as a Case Example. J Empir Res Hum Res Ethics 2: 11-24.

WHO. (2004). Tick-borne diseases – Viruses. . In: World Health Organization, The vector-borne human infections of Europe- their distribution and burden on public health. Monograph: 39-41.

WHO. (2011). Vaccines against tick-borne encephalitis: WHO position paper. Weekly epidemiological record - Relevé épidémiologique hebdomadaire 24(86): 241-256. http://www.who.int/wer.

WHO. (2014a). Tick-borne encephalitis. http://www.who.int/biologicals/vaccines/tick_borne_encephalitis/en/.

WHO. (2014b). Tick-borne Encephalitis Vaccine. Weekly Epidemiological Record - Relevé épidémiologique hebdomadaire 86(24): 241-256.

WHO. (2015). WHO estimates of the global burden of foodborne diseases: foodborne disease burden epidemiology reference group 2007-2015. WHO Library Cataloguing-in-Publication Data. ISBN 978 92 4 156516. pp. 268. http://apps.who.int/iris/bitstream/10665/199350/1/9789241565165_eng.pdf?ua=1.

Wikipedia. (2016). Fourier analysis. https://en.wikipedia.org/wiki/Fourier_analysis#Fourier_series.

Wildtech. (2010). Our Priority and Additional Pathogens. Wildtech Project Website. http://www.wildtechproject.com/wildtech/node/21.

Willadsen P., Bird P., Cobon G.S., Hungerford J. (2005). Commercialization of a recombinant vaccine against Boophilus microplus. Parasitology 110: 43-50.
Wilson M.I., Adler G.H., Spielman A. (1985). Correlation between abundance of deer and that of the deer tick Ixodes dammini (Acari: Ixodidae). Ann Entomol Soc Am 7: 172–176.
Wint W., Alexander N. (2013). Using Habitats, Expert opinion and quantitative spatial modelling to fill in gaps in Administrative Unit distributions Phases 1 and 2. . Report by Environmental Research Group Oxford to the European Centre for Disease Prevention and Control (ECDC) Stockholm. .
WIV-ISP. (2011). Rapport peillaboratoria Lyme ziekte, 2010. https://www.wiv-isp.be/epidemio/epinl/plabnl/plabannl/10_041n_v.pdf.
WIV-ISP. (2015). Neem deel in het tellen van tekenbeten op mensen in België. Aantal gemelde tekenbeten in België gedurende de geselecteerde periode. . https://tekennet.wiv-isp.be/.
Wohlfarth K., Niederstraßer O., Dobler G., Mertens M., Ulrich R.G., Donoso-Mantke O., Niedrig M., Werdermann S., Petri E. (2009). Seroprevalence for tick-borne encephalitis virus (TBEV) in forestry workers in the federal state of Brandenburg, Germany. X International Jena Symposium on Tick-borne Diseases (IJSTD-X 2009) Abstract V46: p. 70.
Wójcik-Fatla A., Cisak E., Zajac V., Zwolinski J., Dutkiewicz J. (2011). Prevalence of tick-borne encephalitis virus in Ixodes ricinus and Dermacentor reticulatus ticks collected from the Lublin region (eastern Poland). Ticks Tick Borne Dis 2: 16-19.
WorldClim. (2015). Global climate data: current conditions (1950-2000). Free climate data for ecological modeling and GIS. http://www.worldclim.org/download.
Wurm R., Dobler G., Peters M., Kiessig S.T. (2000). Serological investigations of red foxes (Vulpes vulpes L.) for determination of the spread of tick-borne encephalitis in Northrhine-Westphalia. J Vet Med B Infect Dis Vet Public Health 47: 503-509.
Youden W.J. (1950). Index for rating diagnostic tests. Cancer 3: 32-35.
Yun S.M., Song B.G., Choi W., Park W.I., Kim S.Y., Roh J.Y., Ryou J., Ju Y.R., Park C., Shin E.H. (2012). Prevalence of tick-borne encephalitis virus in ixodid ticks collected from the republic of Korea during 2011-2012. Osong Public Health Res Perspect 3: 213-221.
Zanoni M., Ortolani D.B., Bonilauri P., Gelmetti D., Fabbi M., Cordioli P., Alborali L.G. (2009). Encefalite da zecche in un cane. XI Congresso Nazionale S.I.Di.L.V. Parma: 52-53. [in Italian].
Zanotto P.M., Gao G.F., Gritsun T., Marin M.S., Jiang W.R., Venugopal K., Reid H.W., Gould E.A. (1995). An arbovirus cline across the northern hemisphere. Virology 210: 152-159.
Zeimes C.B., Olsson G.E., Hjertqvist M., Vanwambeke S.O. (2014). Shaping zoonosis risk: landscape ecology vs. landscape attractiveness for people, the case of tick-borne encephalitis in Sweden. Parasit Vectors 7: 1756-3305.
Zeman P. (1997). Objective assessment of risk maps of tick-borne encephalitis and Lyme borreliosis based on spatial patterns of located cases. Int J Epidemiol 26: 1121-1129.
Zeman P., Januska J. (1999). Epizootiologic background of dissimilar distribution of human cases of Lyme borreliosis and tick-borne encephalitis in a joint endemic area. Comp Immunol Microbiol Infect Dis 22: 247-260.
Ziegler U., Jost H., Muller K., Fischer D., Rinder M., Tietze D.T., Danner K.J., Becker N., Skuballa J., Hamann H.P., Bosch S., Fast C., Eiden M., Schmidt-Chanasit J., Groschup M.H. (2015). Epidemic Spread of Usutu Virus in Southwest Germany in 2011 to 2013 and Monitoring of Wild Birds for Usutu and West Nile Viruses. Vector Borne Zoonotic Dis 15: 481-488.
Zizi M., Heyman P., Vandenvelde C. (2002). The assessment of human health risks from rodent-borne diseases by means of ecological studies of rodent reservoirs. Mil Med 167: 70-73.
Zlontnik I., Grant D.P., Carter G.B. (1976). Experimental infection of monkeys with viruses of the tick-borne encephalitis complex: degenerative cerebellar lesions following inapparent forms of the disease or recovery from clinical encephalitis. Br J Exp Pathol 57: 200-210.

Zöldi V., Papp T., Rigo K., Farkas J., Egyed L. (2014). A 4-Year Study of a Natural Tick-Borne Encephalitis Virus Focus in Hungary, 2010-2013. Ecohealth.